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Studies on the Molecular Cloning of a Gene for Beta-Glucosidase From a Bacillus Cereus/ Cellulomonas Sp. Hybrid Bacterium Derived Through Protoplast Fusion.

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STUDIES ON THE MOLECULAR CLONING OF A GENE FOR β-GLUCOSIDASE FROM A *BACILLUS CEREUS*/*CELLULOMONAS* SP. HYBRID BACTERIUM DERIVED THROUGH PROTOPLAST FUSION

A Dissertation

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requirements for the degree of
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in

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by

Eun Soo Han
M.S., Louisiana State University, 1983
August, 1986

To my parents

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ABSTRACT

A β-glucosidase gene was cloned from a cellulolytic bacterium, which was obtained by hybrid-formation between Bacillus cereus and a Cellulomonas sp. through protoplast fusion, into Escherichia coli plasmid pBR322 using recombinant DNA techniques. E. coli strain transformants of JM83 harboring this cloned plasmid were able to utilize cellobiose as a carbon source. β-glucosidase activity was assayed in these cells by P-nitrophenyl β-D-glucopyranoside (PNPG) hydrolysis. Hybridization of the cloned DNA fragment with DNA from the hybrid organism was performed by Southern transfer (92) and DNA/DNA dot blot procedures. The hybridization data strongly suggested that the DNA cloned into pBR322 was from the genome of the hybrid organism. A restriction map was constructed by analysis of the cleavage sites of restriction endonucleases that recognize specific hexanucleotide sequences. The size of the cloned DNA fragment was 1.2 kb as determined by agarose gel electrophoresis. The nucleotide sequence was determined by subcloning partial fragment of 1.2 kb DNA into M13 phage and using the dideoxy sequencing method developed by Sanger et al. (85). The DNA sequence showed that the cloned DNA contained an open reading frame probably coding for β-glucosidase activity, though not ending with a translation stop codon. This presumably incomplete open reading frame has a coding capacity for 319 amino acids, that corresponds to a protein with a molecular weight of 38,000 daltons.

INTRODUCTION

Cellulose forms the bulk of the cell wall material of all plants. It is thus the most abundant organic material on earth and one of man's least exploited and most useful natural resources. It is unique among major industrial raw materials since it is renewable and therefore potentially an inexhaustable resource.

In recent years, there has been a considerable increase of interest in the conversion of cellulose materials into sugars. Cellulose is susceptible to degradation by the action of various cellulases which are produced by a wide variety of microorganisms. The final breakdown product of cellulose by enzymatic saccharification is glucose, which can be used as a source of food or chemicals. Initial laboratory studies have concentrated on establishing proper culture conditions for cellulase enzyme production, investigating potential substrates, and determining pretreatment methods and optimum conditions for enzymatic hydrolysis. Nevertheless, the present knowledge of the enzymatic processes involved remains incomplete.

Any commercial process for the enzymatic hydrolysis of cellulose will require a source of high quality but inexpensive cellulase enzyme preparation. Hence, the development of a process technology for the large-scale production of cellulase is a primary objective. Although many microorgansims have the ability to grow on cellulose, only a few produce extracellular cellulases capable of converting crystalline cellulose to glucose. Trichoderma viride cellulase preparations show the highest activity, but the organism produces only low amounts of extracellular β-glucosidase (3). β-glucosidases have been isolated

from various organisms and their characteristics have been studied (2, 111). Recently, recombinant DNA techniques have been used to clone several enzymes of the cellulase complex. A β-glucosidase of Escherichia adecarboxylata was cloned and expressed in E. coli (7) and a β-glucosidase of Aspergillus niger was cloned into the host Saccharomyces cerevisiae (72). β-glucosidase gene from Candida pelliculosa was cloned in Saccharomyces cerevisiae and the sequnce of the gene was determined.

In the present study, cloning of a presumptive β-glucosidase gene from a cellulolytic hybrid of Bacillus cereus and Cellulomonas sp. into plasmid pBR322 is described. Introduction of β-glucosidase plasmid pGS2 into E. coli JM83 allowed the organism to grow on minimal medium with cellobiose as the carbon source. The nucleotide sequence of the gene coding for this β-glucosidase activity was determined and analyzed. This is the first report of the nucleotide sequence of a prokaryotic β-glucosidase gene.

LITERATURE REVIEW

I. Biodegradation of Cellulose.

About 1950, Reese and his coworkers first identified Trichoderma strains which produce an active and well-balanced cellulase complex. Cell-free enzyme preparations capable of completely solubilizing native cellulose usually contain three classes of enzymes: extracellular exoglucanases (cellobiohydrolases), endoglucanases (carboxymethyl cellulases) and intracellular β-glucosidases or cellobiases. Many other organisms having cellulase activity have since been isolated (61) but most of them do not have adequate levels of extracellular cellulase for practical use. Practical saccharification data have been published for Penicillium (8), Sclerotium rolfsii (88), and Thermomonospora (21) cellulases, however most studies have used cellulase from Trichoderma reesei (4, 25, 26, 27, 39, 45, 59). T. lignorum and T. koningii also appear to produce active cellulases. The mutant strains of Trichoderma, QM9123 (60), QM9414 (60), C30 (66) and NG14 (66) are in use for cellulase production and are all descendants from QM6a, originally T. viride later renamed as T. reesei. The advantage of Trichoderma is that it produces a complete cellulase with all the components required for the hydrolysis of crystalline cellulose (58). The disadvantages are that the Trichoderma cellulases have a low specific activity, are inhibited by product (cellobiose) (10, 95) and contain only low levels of cellobiase. Nevertheless, it is the best cellulase complex available today.

Recently, new mutant strains of Trichoderma producing cellulases with higher actvities have been selected, and investigations on strain

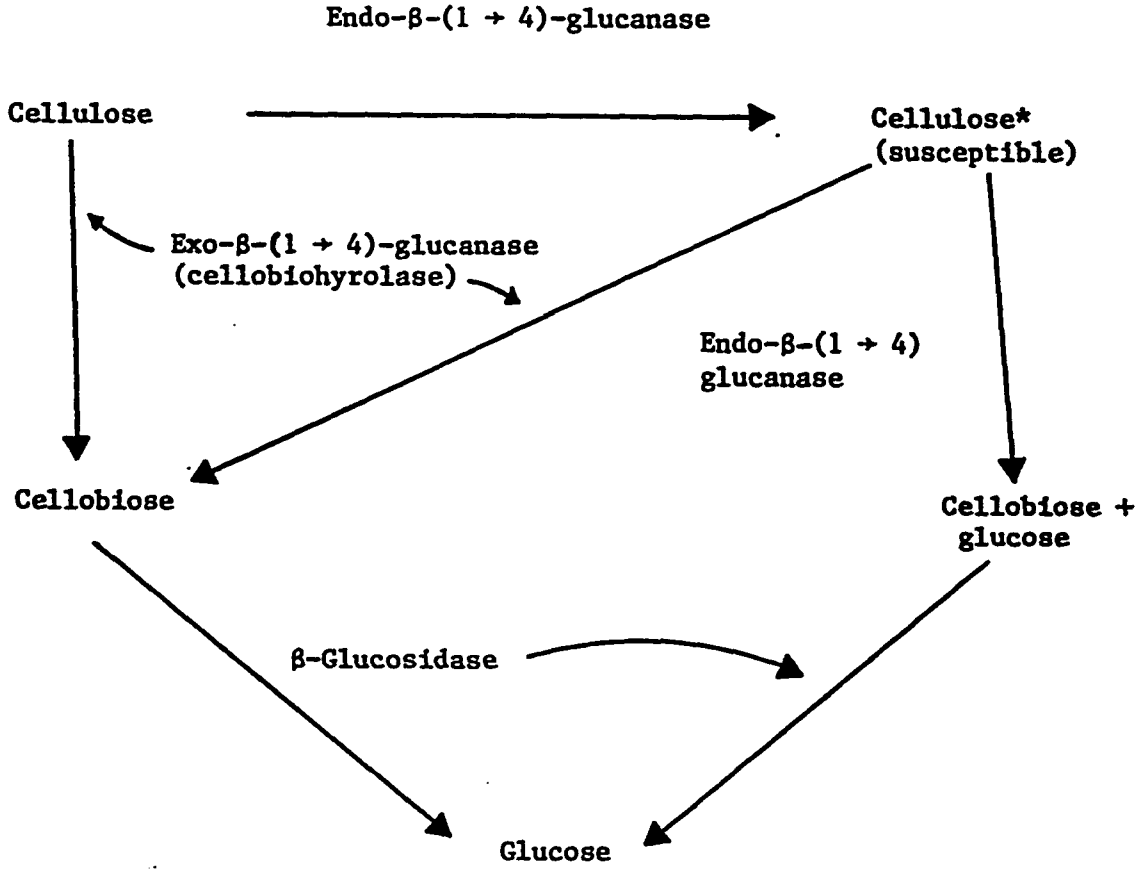
improvement continue in several laboratories (Table 1) (84). The enhanced cellulase mutants have also been obtained from thermophilic actinomyces (79, 90, 97, 110), thermophilic anaerobes (51), and yeasts (18). Many physical and chemical pretreatment methods are being developed in order to improve the hydrolysis rate and product yield (32, 99). The properties of reactive substrates have been studied (5) and susceptibility of cellulosic substrates to cellulase examined (4). It appears that the major factor which controls the difference in the reactivity of the crystalline and amorphous cellulose is the adsorption capacity of endoglucanase on cellulose. The "tightness" of adsorption suggests the quality of binding between the enyzme and substrate. Tighter binding gives better catalysis (47). The continuous process of hydrolysis of cellulose has been attempted in several laboratories (25, 26). Addition of β-glucosidase to the cellulose hydrolysis system enhanced cellulose hydrolysis (114). The mode of hydrolysis is presented in Figure 1 (84). The kinetics of different aspects of hydrolysis of cellulose by cellulase have been studied with respect to cellulose adsorption on the substrate (41, 71), mechanism of reaction per se (86) as well as effect of concentrations of substrates on the inhibition of reaction (39) and deactivation of enzyme (40). Mathematical modeling of the system has been attempted but this has not been entirely successful due to a lack of good understanding of the exact reaction mechanism of the cellulase complex (52).

Table 1. Cellulase production by mutant strains of Trichoderma reesei (84).

Strain	CMC (units/ml)	Filter paper (units/ml)	β-Glucosidases (units/ml)	Productivity (FPU/l/h)	Soluble protein (mg/ml)
QM6a (parent)	88	5	0.3	15	7
QM9414 (Natick)	109	10	0.6	30	14
MCG77 (Natick)	104	11	0.9	33	16
C30 (Rutgers)	150	14	0.3	42	19
NG14 (Rutgers)	133	15	0.6	45	21

Cultures grown 14 days in 10 l fermenters on 6% 2 roll-milled cotton
pH control > 3.0 using 2 N NH_4OH
Enzyme units = µmol glucose produced per min standard assay

Figure 1. Mode of action of cellulase on cellulose-cellulase enzyme system (84).


Endo-β-(1 → 4)-glucanase
Cellulose
Cellulose*
(susceptible)
Exo-β-(1 → 4)-glucanase
(cellobiohyrolase)
Endo-β-(1 → 4)
glucanase
Cellobiose
Cellobiose +
glucose
β-Glucosidase
Glucose

II. β-glucosidases.

1. Occurrence and characteristics of β-glucosidase.

The enzyme β-glucosidase (β-D-glucoside glucohydrolase) catalyzes the hydrolysis of alkyl and aryl β-D-glucosides (e.g., methyl β-D-glucoside and P-nitrophenyl β-D-glucoside) as well as glucosides containing only carbohydrate residues (e.g., cellobiose). The affinity of the enzyme for a particular substrate varies with the nature of its source. β-glucosidase occur ubiquitously in microorganisms such as fungi (63, 113), yeast (19, 113), and bacteria (113) as well as higher organisms (24). Plants contain aryl as well as alkyl β-glucosidases. In plants the pigments of flowers are constituted from the glycosides of various flavones and anthocyanins (106). These glycosides contain a sugar residue and a non-sugar portion termed the aglycon which is often a phenolic derivative. Aryl β-glucosidases would be the essential enzymes for their catabolism. These enzymes are also thought to be involved in conferring resistance on plants against phytopathogens. In the 'fire-blight' disease of pear and apple trees, hydrolysis of the glycoside arbutin (3-hydroxyphenyl β-D-glucoside) by β-glucosidase from the plant results in the release of hydroxyphenol which is toxic to the invading pathogen (24). Among microorganisms several wood-decaying fungi have been extensively investigated for their β-glucosidase activities. The fungi produce β-glucosidases having high affinity for the cellulose-derived cellobiose for complete degradation to glucose. Several yeast strains such as Trichosporon cutaneum and Saccharomyces

cerevisiae (19) have also been shown to have β-glucosidase activities. Only a few bacterial β-glucosidases have been thoroughly investigated (113).

Recently much attention has been focused on β-glucosidases because of the following problems that need to be overcome before the enzymatic hydrolysis of cellulose becomes commercially viable: [1] This enzyme is inhibited by its end product, glucose (31), which accumulates during the reaction and inhibits the hydrolysis of cellobiose. [2] The culture filtrates with cellulases containing extramycelial levels of β-glucosidase which are too low to be used in practice, especially in the case of Trichoderma reesei (3). [3] Due to thermal inactivation, a large proportion of β-glucosidase activity is lost during the reaction (30).

Solutions to these problems have been suggested and include: [a] addition of β-glucosidase in immobilized form to cellulase in order to increase the rate of cellobiose hydrolysis (12, 105), [b] the isolation of mutant organisms which can produce extramycelial β-glucosidase at increased levels (3), and [c] since fructose is a poor inhibitor of the β-glucosidase, the conversion of cellulose or cellobiose derived glucose to fructose to prevent end-product inhibition of β-glucosidase (112).

A summary of the properties of fungal β-glucosidases is given in Table 2 (113). Two different β-glucosidases (classified on the basis of their cellular location) are induced when the yeast Saccharomyces cerevisiae is grown on cellobiose (42, 44). Intracellularly located enzymes possess little affinity for cellobiose and methyl β-D-glucoside. Cell surface located enzymes, have broader substrate specificities with

Table 2. Properties of fungal β-glucosidases (113).

Source	pH		Temperature (°C)		K_m (mM)		Molecular weight	Subunit size	Carbohydrate content (%)
	Optimum	Stability	Optimum	Stability[a]	PNPG	Cellobiose			
S. cerevisiae	6.8	n.d.	n.d.	n.d.	0.095	n.d.	313 000	n.d.	n.d.
S. cerevisiae	6.4-6.8	6.0-8.0	45	45	n.d.	n.d.	300 000	n.d.	n.d.
S. fragilis	5.7-6.2	5.0-7.0	n.d.	50	0.11	n.d.	n.d.	n.d.	n.d.
Candida guilliermondii	6.8	n.d.	37	45	0.125	n.d.	48 000	n.d.	n.d.
A. oryzae	4.0-5.0	5.0-7.0	n.d.	n.d.	n.d.	n.d.	218 000	2	n.d.
A. fumigatus	4.0-5.0	n.d.	n.d.	n.d.	0.88	0.84	340 000	2	n.d.
A. phoenicis	4.3	5.5	n.d.	70	44.0	0.75	n.d.	n.d.	n.d.
A. fumigatus	5.0	n.d.	n.d.	n.d.	6.3	n.d.	41 000	n.d.	8
A. wentii	1.5-5.0	n.d.	n.d.	n.d.	n.d.	n.d.	170 000	n.d.	22
T. viride	5.0	n.d.	n.d.	n.d.	n.d.	2.5	76 000	n.d.	n.d.
T. reesei	6.5	n.d.	n.d.	40	n.d.	3.3	98 000	n.d.	n.d.
T. reesei	4.8	n.d.	n.d.	n.d.	3.5	1.9	n.d.	n.d.	n.d.
Thermoascus aurantiacus	5.0	6.0-8.0	70	70	0.52	n.d.	85 000	n.d.	33
Botryodiplodia theobromae	n.d.	n.d.	n.d.	n.d.	0.33	n.d.	350 000	8	n.d.

[a] Temperature at which rapid inactivation occurs; n.d. not determined

a high affinity for cellobiose as well as for alkyl and aryl β-D-glucosides. These two enzymes have similar pH optima and molecular weights. The β-glucosidase located at the cell surface presumably enables the yeast cells to utilize β-glucosides as a source of carbon and energy. The function of the intracellularly located enzyme is not understood. Recently, the intracellular β-glucosidase in a strain of S. cerevisiae was found to remove the terminal glucose molecules from the oligosaccharide Glc_3 Man_9 $GlcNAc_2$ (46). It has been proposed that this enzyme may be utilized in glycoprotein synthesis, or a role in the processing of oligosaccharides after their transfer from dolichyl pyrophosphate to proteins (56). Another interesting β-glucosidase was found in a different strain of S. cerevisiae (19). This enzyme is active on both alkyl and aryl β-D-glucosides, and shows higher rates of hydrolysis with increasing chain length of the aglycon group. For instance, it catalyzes the hydrolysis of n-decyl β-D-glucoside at a rate six times higher than it catalyzes the hydrolysis of methyl β-D-glucoside. In general, yeast β-glucosidases possess low activity towards cellobiose and are highly active on aryl β-D-glucosides. These enzymes have a pH optimum between 5.7 and 6.8 and they are unstable at temperatures higher than 50°C. Molecular weights of these enzymes range around 300,000 daltons. However, a β-glucosidase with similar enzymatic properties was purified from the yeast Candida guilliermondii, and was found to have a molecular weight of 48,000 (80).

In contrast, β-glucosidase from the black Aspergilli is more active on cellobiose (96) than ones from yeast. The pH optimum of Aspergillus β-glucosidase is 4.0 to 5.0, which is much lower than β-glucosidase from

yeast. Furthermore, the A. phoenicis and A. niger β-glucosidases have high thermal stability (96). They are rapidly inactivated only at temperatures higher than 65°C. The molecular weights of A. fumigatus and A. oryzae β-glucosidases are 340,000 and 218,000, respectively. Each enzyme is possibly composed of two subunits (63, 82). Many disaccharides are good substrates for Aspergillus β-glucosidases. Some Aspergillus β-glucosidases have been found to be glycoproteins containing glucosamine and the neutral sugars glucose and mannose (63, 81, 82). The β-glucosidase from A. wentii has been shown to have 20% w/w carbohydrate (53).

The β-glucosidases from Trichoderma reesei are not well characterized although they have been shown to be highly active on cellobiose as well as on aryl β-D-glucosides. At early stages of growth, the enzyme is bound to the mycelium but after lysis of the mycelium, which occurs in the later stages of growth, it is released into the culture medium. The β-glucosidase secreted by Trichoderma reesei is the most stable of the known β-glucosidases. The enzyme preparation from the C30 strain loses little activity after 28 days at 50°C and pH 4.8 (74). The optimum pH for β-glucosidase activity in the culture fluid of T. reesei was pH 5.0. Recently, an intracellular β-glucosidase has been isolated from the T. reesei strain QM9123 (43). This enzyme had a pH optimum of 6.5 but was not stable at temperatures above 40°C. The function of this intracellular enzyme in regard to cellulase activity is unknown. It may be involved in the induction of cellulase since it catalyzes the hydrolysis of sophorose (km = 6.7 mM) which is a powerful inducer of cellulase (57).

A characteristic of β-glucosidases from the Aspergillus and Trichoderma genera is that they are inhibited by glucose. Competitive (12, 112) and non-competitive (31) enzyme inhibition of β-glucosidase have been reported from glucose but the mechanism whereby this occurs is not clear.

Other fungal β-glucosidases which have been studied from thermophilic microorganisms. The enzyme isolated from Thermoascus aurantiacus has pH and temperature optima of 5.0 and 70°C, respectively (101). But the optimum pH for enzyme activity and enzyme stability were different. In Sporotrichum (Chrysosporium) termophile, two distinct β-glucosidases have been found (65). One of the enzymes which has a molecular weight of 440,000 shows activity against only aryl β-glucosides while the smaller enzyme (molecular weight 40,000) has cellobiase activity. Both enzymes were associated with the mycelium and had similar pH optima, 5.6-6.3; temperature optimum, 50°C; and temperature stability, 47-48°C. Some interesting kinetics have been reported for Botryodiplodia theobromae β-glucosidase (103). When P-nitrophenyl β-D-glucoside (PNPG) was used as a substrate, the enzyme was activated to the extent of 10-20% by low concentrations of cellobiose but it was inhibited by cellobiose at higher concentrations (≥1 mM). Glucose was a competitive inhibitor whereas maltose was a non-competitive inhibitor. Glycerol activated the enzyme by 20%. Aging of this enzyme resulted in its dissociation from a molecular weight of 350,000-380,000 daltons to 45,000-47,000 daltons (103). It was shown by electron microscopy that the high molecular weight form of this enzyme

was an octamer. This enzyme was further dissociated into polypeptides of molecular weight 10,000-12,000, however, these polypeptides were found to be inactive.

The β-glucosidase from anaerobic bacterium Clostridium thermocellum has recently been characterized (1). The enzyme was located in the periplasmic space, which is the space between the cell wall and cell membrane. The pH and temperature optima of this enzyme were 6.0 and 65°C, respectively. The enzyme was stable for 7 hrs at 60°C. The enzyme had greater affinity for the aryl β-D-glucosides.

There are reports of characterization of several bacterial β-glucosidases. Their properties are summarized in Table 3 (34, 113).

2. Mechanism of enzymatic hydrolysis by β-glucosides.

The exact mechanism of enzymatic hydrolysis by β-glucosidase is unknown. Studies with the enzyme from Aspergillus wentii suggest the involvement of a carboxylate anion in the catalytic activity (9). Affinity labeling of the active site of β-glucosidase with ^{3}H-conduritol-B-epoxide, which is structurally related to glucose, followed by proteolytic cleavage of β-glucosidase enzyme with pepsin showed that the radioactivity was bound as an ester of (+)chiro-inositol to aspartic acid (9). A similar result was obtained when the enzyme was labeled with the active site-directed inhibitor D-glucal (55). When the enzyme was incubated with ^{3}H-labeled P-nitrophenyl-2-deoxy-D-glucose and subsequently denatured, it was also found that this labeled substrate was bound to an aspartate side chain (78). It appears that β-glucosidases from various sources form an essential carboxylate anion since they are all inhibited by conduritol-B-epoxide (54). Modification of the hydroxyl groups in glucose results in a drastic decrease in

Table 3. Properties of bacterial β-glucosidases (34, 113).

Source	pH		Temperature (°C)		K_m (mM)		Molecular weight
	Optimum	Stability	Optimum	Stability[a]	PNPG	Cellobiose	
Cl. thermocellum	6.0	n.d.	65	60	2.1	83	50 000
Ac. cellolyticus	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	81 000
B. succinogenes	6.8	n.d.	n.d.	n.d.	n.d.	43	n.d.
E. herbicola	6.0-7.5	6.0-7.5	n.d.	50	n.d.	n.d.	122 000
A. faecalis*	6.8	6.5-7.8	n.d.	55	0.125	n.d.	110 000

[a] Temperature above which rapid inactivation occurs; n.d., not determined

*(34)

β-glucosidase activity (78). The exact role of the hydroxyl groups on the substrate is not known, but they may be necessary for correct positioning of the substrate in the active site, which is necessary for bond cleavage between carbon atom 1 and the glycosyl oxygen. Although glucose is a potent inhibitor of β-glucosidase, 3-O-methyl-D-glucose has no effect on the activity of the enzyme from T. reesei (112).

III. Recombinant DNA Technology for Production of Cellulolytic Enzymes.

Many attempts have been made for quantitative and qualitative improvements in cellulases from microbial sources. Mutagenesis (37, 66), DNA transfer by protoplast fusion (29, 33) and conventional recombinant DNA techniques (77, 91) have been used. Since cellulases are multienzyme systems, it is not likely that a single DNA region would code for the entire cellulase complex, that would be expressed in a different host environment. The method of protoplast fusion could be advantageously used to overcome this difficulty since it allows the transfer of large segments of DNA from one organism to another (69). Intrageneric and intergeneric protoplast fusions of bacteria and fungi have been reported (6, 22, 87).

Studies on the transfer of genes coding for cellulase by protoplast fusion have been made between Cellulomonas sp. and Bacillus cereus (33) as well as Bacillus subtilis (29). Stable organisms sharing the genetic markers of both parental strains were obtained by PEG-induced protoplast fusion. The hybrids exhibited characteristics of cellulose degradation. In addition, extracellular β-glucosidase was detected in the fusion products. But it was recognized that the cellulase produced by the hybrid organisms did not have enough activity for commercial use.

A different approach to increased cellulase production is to clone the gene or genes coding for cellulase on a recombinant DNA plasmid. The expression of gene(s) might then be modified by current molecular genetic techniques.

Through the combined efforts of molecular biologists, biochemists, and bioengineers, the complicated story of cellulases is beginning to be understood. Research in the U.S. (89, 108), Canada (107), Finland (100), and France (16) has resulted in molecular cloning and expression of genes coding for specific cellulases. All the important fungal enzymes have been cloned by Shoemaker *et al.* at Cetus Corporation and elsewhere (Table 4) (20). Endo-β-glucanases of bacteria such as *Clostridium thermocellum*, *Bacillus subtilis*, *Bacillus polymyxa*, *Thermomonospora* sp., and *Cellulomonas fimi* have also been cloned by several groups (20). For example, Whittle *et al.* (107) reported the successful molecular cloning of a cellulase gene from *Cellulomonas fimi* on plasmid pBR322 in *E. coli*. They detected active cellulase in extracts of one *E. coli* transformant. Gene fusions were also contructed by Skipper *et al.* (91) between a yeast expression plasmid and a *Cellulomonas fimi* DNA fragment encoding an endo-1,4-β-D-glucanase or carboxymethyl cellulase. Carboxymethyl cellulase activity was secreted by yeast transformed with the recombinant plasmids. The cloning of the *B. subtilis* DLG β-1,4-glucanase gene in *E. coli*-*B. subtilis* shuttle vector, pPL1202 was carried out by Robson and Chambliss (77). This enzyme is capable of degrading both carboxymethyl cellulose and trinitrophenyl carboxymethyl cellulose, but not crystalline cellulose

Table 4. Fungal cellulase genes cloned (20).

Enzyme	Organism
Cellobiohydrolase I	*Trichoderma reesei*
Cellobiohydrolase I	*T. reesei*
Cellobiohydrolase II	*T. reesei*
Endoglucanase	*T. reesei*
β-glucosidase	*Aspergillus niger*

substrates. The variable expression and fate of the enzyme in E. coli C600 SF8 and B. subtilis PSL1 were also studied by them (77).

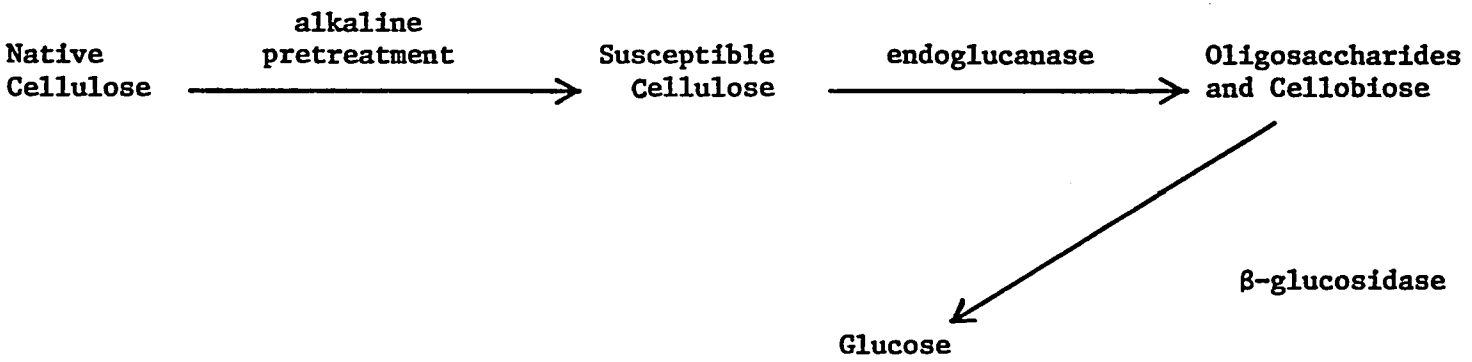
The availability of cloned cellulase genes has opened up new possibilities. These clones offer opportunities for a more extensive study of the individual enzymes. Furthermore, as isolated genes become available, modification and expression of these genes in the same or different microorganisms becomes possible. It was encouraging when fungal cellulase clones were shown to be secreted efficiently into the growth medium by yeast (20). Thus it has become possible to construct new organisms that produce a single cellulolytic enzyme.

It is possible to obtain glucose from cellulose by the combined activities of only two of the enzymes of the cellulase complex if cellulose is properly pretreated prior to degradation (Figure 2). The required enzymes are endoglucanase and β-glucosidase. Fungal and bacterial endoglucanase genes have been cloned and extensively studied (20). However only a few reports have been published about the molecular cloning of β-glucosidase genes. β-glucosidase genes from Aspergillus niger (72), Escherichia adecarboxylata (7) and Candida pelliculosa (48) have been cloned but only the gene from Candida pelliculosa has been sequenced (48).

IV. Biotechnological Applications of β-glucosidase.

Ghose and Pathak (28) listed fourteen applications of cellulases, including solubilizing brewing and other raw materials, and removing soybean seed coat. Other applications include garlic processing and mushroom softening. Cellulase, perhaps containing phytase activity, may prove to be a useful feed supplement, especially for poultry and pigs.

Figure 2. Enzymatic way to obtain glucose from cellulose.


Native
Cellulose
alkaline
pretreatment
Susceptible
Cellulose
endoglucanase
Oligosaccharides
and Cellobiose
β-glucosidase
Glucose

As mentioned earlier, the Trichoderma cellulase enzyme system has been studied extensively and has insufficient β-glucosidase activity for the practical saccharification of cellulose. Hence, some problems involved in the commercially available enzymatic hydrolysis of cellulose are related to β-glucosidase. Reese et al. (96) found that the black Aspergilli (A. niger and A. phoenicis) were superior producers of β-glucosidase. When Trichoderma cellulase preparations were supplemented with β-glucosidase from Aspergillus during practical saccharifications, glucose was the predominant product and the rate of saccharification significantly increased. The stimulatory effect of β-glucosidase appeared to be due to the removal of inhibitory levels of cellobiose.

The enzyme β-glucosidase could be recovered and reused by immobilizing the enzyme. Enzymes bound to insoluble, solid matrices promise to be of considerable value both in industry and in research, because they are readily recoverable for reuse, and because they often possess greater stability than the native forms. Sternberg and Bissett (12) found that β-glucosidase of Aspergillus phoenicis QM329 immobilized on chitosan, using glutaraldehyde, when compared with free β-glucosidase, exhibited a similar pH optimum activity but higher activity at lower pH values, and displayed improved thermal stability although only 1-10% of the enzyme activity was retained. On the contrary, Sundstrom et al. (98) reported that β-glucosidase from Aspergillus phoenicis immobilized by sorption on controlled-pore alumina was found to retain 90% activity. Both soluble and immobilized β-glucosidase have similar thermal stability, with significant

deactivation occurring above 65°C. When this immobilized β-glucosidase was used together with Trichoderma reesei cellulase to hydrolyze cellulosic material, increased yields of glucose and greater conversions of cellulose to glucose were observed. When almond β-glucosidase was immobilized on the cation exchange resin DP-1, retention of activity was near 50% at a pH of 5.0 and it showed less heat stability than the free enzyme (105). Recent studies by Woodward and Wiseman (113) showed that when Aspergillus niger β-glucosidase was treated with glutaraldehyde and then immobilized onto concanavalin A-sepharose, there is about 70% retention of enzyme activity and it has increased thermal stability at 65°C compared with the free enzyme. The properties of immobilized β-glucosidase preparations are shown in Table 5 (113).

Immobilized β-glucosidase may also be used in clinical therapy. In Gaucher's disease, difficiency of β-glucosidase has been demonstrated in a variety of cells and tissues (93, 102). Suitable β-glucosidase may be immobilized into liposomes which are lipid capsule containing lipid bilayers in alternation with aqueous layers (109). The liposomes could be taken up, mainly into the liver and spleen by endocytosis mechanisms, and the enzyme is then liberated into the lysosome to catalyze the breakdown of the glucocerebrosides (109). Immobilization and chemical modification could change the properties of the β-glucosidase with the purpose of increasing its stability toward temperature, pH and proteolytic cleavage.

Recombinant DNA technology can also be applied to improve the enzymatic properties of β-glucosidase. Cloning of β-glucosidase genes into suitable plasmids could offer opportunities for more extensive

Table 5. Properties of immobilized β-glucosidases (113).

Source	Carrier material	Retention of activity (%)	pH Optimum	Temperature stability	K_m cellobiose (mM)
A. phoenicis	None	100	4.8	Normal	0.8
	Chitosan	1-10	4.8	Increased	3.7
	Alumina	90	3.5	Unchanged	2.7
Sweet almonds	None	100	5.6	Normal	117
	Amberlite	50	5.0	Decreased	216
	Cellulose	60	5.5	Increased	n.d.
	Fibroin	47	5.7	Increased	n.d.

n.d., not determined

studies of this enzyme. The cloned gene could be modified further or expressed in the same or different microorganisms. *In vitro* modification of the gene may yield an enzyme which is not inhibited by glucose, that would allow more flexible use of this enzyme.

MATERIALS AND METHODS

I. Bacterial Strains.

The organism used in these studies was obtained by fusion of protoplasts in the presence of polyethyleneglycol 6000 between *Bacillus cereus* (Spo^+ Asp^- Tet^R) and *Cellulomonas* sp. (Thi^- Bio^-) in our laboratory. The hybrid organism possesses the genotype of Spo^+ Tet^R (29, 33).

Escherichia coli strains used were JM83 and JM101. Their genotypes were: i) JM83; *ara*. Δ(*lac-proAB*), *rpsL* (=*strA*), φ80, *lacZ*ΔM15 (115), and ii) JM101; *sup*E. *thi*. Δ(*lac-proAB*), [F',*tra*D36, *pro*AB. *lacI*^{q} ZΔM15] (115). The *E. coli* strain JM83 was obtained from Dr. Braymer's culture collection in the Department of Microbiology, Louisiana State University. The other *E. coli* strain JM101 was obtained from Dr. R. Montelaro from the Department of Biochemistry, Louisiana State University.

II. Maintenance of Organisms.

The hybrid organism was maintained on slants of agar with 2 g cellobiose (Sigma) per liter of distilled water and salts containing 0.45 mM $(NH_4)_2SO_4$, 1 mM K_2HPO_4, 0.4 mM NaH_2PO_4, 0.492 mM $MgCl_2 \cdot 6H_2O$, 0.0204 mM $CaCl_2 \cdot 2H_2O$, 0.4 nM $CuSO_4 \cdot 5H_2O$, 0.0169 μM $CoCl_2 \cdot 6H_2O$, 0.510 μM $MnCl_2 \cdot 4H_2O$, 0.33 μM $ZnSO_4 \cdot 7H_2O$ and 1.8 μM $FeSO_4 \cdot 7H_2O$. The final pH of the medium was 6.7.

E. coli strain JM83 was maintained on L agar slants containing 10 g Bacto-tryptone (Difco), 5 g Bacto-yeast extract (Difco), 10 g NaCl and 15 g agar (Difco) per liter of distilled water. The final pH was 7.4. Ampicillin was added to a final concentration of 40 μg/ml when necessary.

E. coli strain JM101 was maintained on a minimal agar medium containing 6 g NaH_2PO_4, 3 g Na_2HPO_4, 0.5 g NaCl, 1 g $(NH_4)_2SO_4$, and 15 g agar per liter of distilled water. After sterilization and cooling the following constituents were added to the final concentration: 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 0.2% glucose and 0.0001% thiamine.

III. Media.

The growth medium for the hybrid bacterium contained 8 g nutrient broth (Difco) supplemented with 2 g yeast extract per liter of distilled water.

The E. coli strain JM83 was grown on a medium containing the same constituents as the maintenance medium without the agar (LB). The transformed clones of E. coli were grown on minimal cellobiose medium of the following composition: 2 g cellobiose, 0.2 g yeast extract and all the salts present in the maintenance medium for the hybrid organism per liter of distilled water. The final pH was 7.0. SOB consisted of the following: 10 g tryptone, 5 g yeast extract, 0.6 g NaCl, 0.4 g KCl, 2 g $MgCl_2$, and 2.5 g $MgSO_4$ per liter of distilled water. Magnesium salts were filter sterilized and added after autoclaving. The final pH was 7.2. SOC was SOB medium supplemented with 3.6 g glucose per liter. Modified MacConkey agar was Bacto MacConkey agar base (17.0 g Bacto peptone, 3.0 g proteose peptone, 1.5 g Bacto-bile salts No. 3, 5.0 g NaCl, 13.5 g Bacto-agar, 0.03 g Bacto-neutral red, and 0.001 g Bacto-crystal violet, final pH 7.0, Difco) supplemented with 10 g cellobiose per liter of distilled water. λym broth consisted of 10 g Bacto-tryptone, 2.5 g NaCl, 2 g maltose, and 1 g yeast extract per liter of distilled water.

The E. coli strain JM101 was grown on the following media (per liter): i) 1xYT: 8 g tryptone, 5 g yeast extract, and 5 g NaCl; ii) 2xYT: 16 g tryptone, 10 g yeast extract, and 5 g NaCl.

For solid media, 15 g of Bacto-agar (Difco) were added per liter. Ampicillin, tetracycline and kanamycin sulfate were used whenever required at final concentrations of 40 μg/ml, 15 μg/ml and 25 μg/ml, respectively.

IV. Vectors.

The plasmid vector used in these studies was pBR322 (13). The phage vector utilized was M13 mp9 (64). pBR322 was obtained from the Department of Microbiology culture collection, Louisiana State University. M13 mp9 was obtained from R. Montelaro, Louisiana State University.

V. λTn5.

Bacteriophage lambda b221 rex::Tn5 CI857 Oam8 Pam29 (83) was obtained from Dr. R. Gayda, Louisiana State University.

VI. Enzymes Used in Molecular Cloning.

Restriction endonucleases, T_4DNA ligase, bacterial alkaline phosphatase, T_4DNA polymerase I were purchased from Bethesda Research Laboratories (B.R.L.) and the large fragment of DNA pol I (Klenow) was purchased from Boehringer Mannheim. T_4DNA ligase and T_4DNA polymerase I used for M13 cloning were purchased from P.L. Biochemicals. Enzymes were used according to the supplier's recommendations.

VII. Methods of DNA Preparation.

1. Isolation of chromosomal DNA from Bacillus-Cellulomonas hybrid.

A loopful of the spores from the slant was suspended in 1 ml of water and heat shocked at 70°C for 15 minutes, cooled to ambient temperature and then inoculated into 10 ml of growth medium. This culture was incubated overnight at room temperature without shaking and then transferred into 90 ml of fresh growth medium. After 3 hrs incubation at 30°C with shaking (200 rpm), the culture was transferred to 900 ml of fresh medium in 2 l flask and incubated for 4 hrs at 30°C with shaking (200 rpm). The cells were harvested by centrifugation at 9,000 rpm in a Sorvall GSA rotor for 30 minutes at 4°C. The cellular wet weight was estimated and the cells were resuspended in saline-EDTA (0.15 M NaCl, 0.1 M EDTA, pH 8.0); one ml of solution was added per gram of wet weight. Two mg of lysozyme were added for each 1 ml of the cells suspended in saline-EDTA. The mixture was then incubated at 37°C for 20 minutes without shaking. Lysis was effected by addition of 10 ml SDS (1%) with 0.1 M Tris-HCl at pH 8.0 and 0.1 M NaCl per 1 ml of mixture. The mixture was frozen at -20°C overnight and thawed at 65°C. The freeze-thaw sequence was repeated once more. An equal volume of buffer (T.E.S.: 0.05 M Tris-HCl, pH 8.0; 5 mM EDTA, pH 8.0; 0.05 M NaCl) saturated phenol (80%) was added to the lysed suspension. The resulting emulsion was incubated at 4°C with occasional shaking for 20 minutes and centrifuged at 12,000 rpm for 20 minutes in the Sorvall centrifuge. The upper aqueous phase containing the DNA was carefully pipetted off into a tube. The DNA was precipitated by the addition of 1/10 volume of sodium acetate (3 M, pH 4.8) and 2 volumes of cold

ethanol, followed by centrifugation at 10,000 rpm (GSA rotor) for 10 minutes at 4°C. The DNA pellet was washed with 80% ethanol. The DNA was resuspended in a T.E. buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA, pH 8.0) with 20 µl RNase (10 mg/ml) per one ml suspension, followed by incubation at 37°C for 30 minutes. Then pronase was added to the solution at a final concentration of 500 µg/ml and the solution was incubated for another 30 minutes at 37°C. The DNA solution was further extracted once with an equal volume of T.E. buffer saturated phenol, once with phenol:chloroform (1:1), and three times with water-saturated ether. Residual ether was removed by heating the mixture at 70°C for 10 minutes. After cooling the solution to room temperature, 1/10 volume of 3 M sodium acetate, pH 4.8, was added to the solution and the DNA was again precipitated with 2 volumes of cold ethanol, and -20°C overnight sitting, followed by centrifugation at 12,000 rpm (GSA rotor) for 15 minutes at 4°C. The DNA pellet was washed with 80% cold ethanol, and dried *in vacuo*. Finally, the DNA was resuspended in T.E. and the concentration was determined by measuring the absorbance at 260 nm.

2. Plasmid DNA isolation.

Two ml of an overnight culture of cells grown in LB with 40 µg/ml ampicillin at 37°C with agitation (250 rpm) were used to inoculate in a 2 liter flask with 1 liter of LB containing 40 µg/ml ampicillin. The culture was incubated at 37°C with shaking (250 rpm) until the cell density reached 100 Klett units (this took approximately 6 hrs). Then 150 mg/l of chloramphenicol were added to the culture and incubation was continued overnight at 37°C with shaking to amplify plasmid DNA. The cells were harvested by centrifugation for 6 minutes at 6,000 rpm to

yield a pellet. The cells were resuspended in 5 ml of a solution of 25% sucrose-50 mM Tris-HCl, pH 8.0. Ten mg of lysozyme were dissolved in 1 ml of a solution of 25% sucrose-50 mM Tris-HCl, pH 8.0 and 0.5 ml of this solution was added to the cell suspension. The suspension was incubated for 5 minutes on ice, and then 0.5 ml of 0.25 M EDTA, pH 8.0, was added. Incubation was continued for another 5 minutes on ice. Next, 5 ml of Triton-lytic solution containing 50 mM Tris-HCl, pH 8.0; 62 mM EDTA, pH 8.0; and 0.4% Triton X-100 were added quickly to the mixture, which was stirred once, and allowed to stand at room temperature until most cells lysed. Cellular debris was pelleted by ultracentrifugation at 45,000 rpm for 45 minutes at 15°C in a Beckman type 75Ti rotor. The supernatant solution was transferred into a plastic centrifuge tube. One gram of cesium chloride (CsCl) and 0.1 ml of a solution of 5 mg/ml ethidium bromide were added per ml of the supernatant fluid. Protein debris was then removed by centrifugation at 10,000 rpm (Sorvall SS34 rotor) for 20 minutes. The supernatant solution was loaded into plastic quick-seal tubes and placed in a VTi65 rotor. The gradients were centrifuged to equilibirum at 49,000 rpm for 20 hrs at 15°C (Beckman ultracentrifuge Model L5-65B). Covalently closed circular plasmid DNA was detected with long-wave ultraviolet light (366 nm). Plasmid-containing bands were collected by puncturing the side of the tubes with a syringe with a #21 gauge needle. In order to remove ethidium bromide the plasmid DNA in CsCl solution was partitioned 5 times with water-saturated butanol. Two volumes of water were then added to dilute the solution and 6 times the original volume of ethanol were added to precipitate DNA. The solution was kept at

-20°C overnight. The DNA was pelleted by centrifugation at 10,000 rpm for 10 minutes at 4°C and the pellet was washed with 80% cold ethanol. The DNA was dried *in vacuo* and resuspended in T.E. buffer.

For rapid screening of plasmid DNA, small quantities of plasmid DNA were isolated by the method of Birnboim and Doly (11) with some modification. Cells containing plasmids were grown overnight in 5 ml of LB with 40 µg/ml ampicillin at 37°C with shaking (200 rpm). One and one-half milliliters of this culture were transferred in a microcentrifuge tube and centrifuged for 30 seconds. The supernatant fluid was removed and 110 µl of a solution containing 25 mM Tris-HCl (pH 8.0), 5 mM glucose, 10 mM EDTA, and 2 mg/ml lysozyme were added to the pellet. The suspension was incubated for 30 minutes on ice. Then, 220 µl of a solution with 0.2 N NaOH and 1% SDS were added and then mixed by inverting tube until the lysate was clear and slightly viscous. The lysate was kept on ice for 5 minutes after which 165 µl of 3 M sodium acetate (pH 4.8) were added to the solution which was mixed until a clot of chromosomal DNA was formed. The tubes were then placed on ice for 60 minutes. The cellular debris was sedimented by centrifugation for 5 minutes. The clear supernatant layer (400 µl) was removed and transferred to a second microcentrifuge tube. One ml of cold ethanol was added to the solution which was held at -70°C for 30 minutes. The plasmid enriched DNA was pelleted by centrifugation for 10 minutes and the supernatant fraction was discarded. The pellet was dissolved in 100 µl of a solution containing 0.1 M sodium acetate and 0.05 M Tris-HCl, pH 8.0. Two volumes of cold ethanol were added to the mixture

which was held at -70°C for 30 minutes. The DNA was sedimented by centrifugation for 10 minutes, washed with 80% ethanol, and dried *in vacuo*. The DNA pellet was resuspended in T.E. buffer.

3. Isolation of M13 RF DNA.

Five hundred ml of 2xYT medium in a 2 l flask were inoculated with 1 ml culture of host cells (JM101) and 1 ml M13 phage suspension and incubated at 37°C for 16 to 24 hours with shaking (The inoculum of JM101 was prepared as follows: 50 ml of 2xYT medium in a 250-ml flask were inoculated with 1 ml of fresh overnight JM101 culture and agitated for 2 hrs at 37°C). An overnight culture of JM101 infected by M13 phage was then divided between two 250 ml polypropylene centrifuge bottles and centrifuged for 15 minutes at 6,000 rpm in a Sorvall GSA rotor. The supernatant fraction was discarded and a solution containing 25 mM Tris-HCl (pH 8.0), 10 mM EDTA and 50 mM glucose was added (3.6 ml per tube) to the pellet. The pellet was then broken up with a 10 ml pipet and transferred to a 50 ml centrifuge tube. The cells were resuspended with a vortex mixer. Another 0.4 ml of above solution with 50 mg/ml lysozyme (freshly prepared) was added to the mixture which was agitated with a vortex mixer. The suspension was held for 10 minutes at room temperature. Eight ml of solution of 0.2 N NaOH and 1% SDS (fresh) were added to the suspension which was mixed gently by inverting the tube several times. The tube was held on ice for 10 minutes, and 6 ml ice cold solution containing 3 M potassium acetate and 2 N acetic acid, pH 4.8, were added to it. The suspension was then mixed by inversion again and held a further 10 minutes on ice. The mixture was centrifuged for 10 minutes at 15,000 rpm in a Sorvall centrifuge (SS34 rotor). The

clear supernatant fraction (∿17 ml) was transferred to a fresh tube. Six-tenth of a volume of isopropanol at room temperature was added to the supernatant solution which was mixed and held for 5 minutes at room temperature. The mixture was centrifuged for 5 minutes at 10,000 rpm and the supernatant fraction was discarded. The pellet was washed with 10 ml of 70% ethanol at room temperature by vortex mixing and centrifuging for 5 minutes, and discarding the supernatant fraction. The pellet was dried in vacuo. Next, 3 ml solution of 25 mM Tris-HCl (pH 8.0) with 10 mM EDTA were added to the pellet and the pellet was resuspended by extensive agitation with a vortex mixer. When the pellet was completely resuspended, the tube was centrifuged briefly to collect the liquid, and the suspension was transferred to a tube containing 3.40 g of CsCl and 0.5 ml of 10 mg/ml ethidium bromide. The tube was inverted several times to mix and dissolve the CsCl and the solution was loaded into a Beckman VTi65 polyallomer centrifuge tube. The remainder of the tube was filled with a solution made from the same components as above. Tubes were balanced, heat sealed and centrifuged for 24 hrs at 45,000 rpm in a Beckman VTi65 rotor. The rotor was decelerated to approximately 8,000 rpm with the brake, then the brake was turned off and the rotor was allowed to stop without braking to avoid disturbing the gradient. The M13 RF DNA band was detected by long-wave U.V. light. Chromosomal DNA was found to band one to 1.5 cm above M13 RF DNA. The M13 RF DNA band was withdrawn from the side of the tube with a number 21 needle and syringe. The volume of the isolated DNA band was measured and the RF DNA was extracted 5 times with isoamyl alcohol to remove ethidium bromide. The final aqueous volume was measured, the solution

was transferred to a siliconized 25 ml corex tube and diluted with H_2O to at least 3.2 times the original volume. DNA was precipitated by the addition of 2 times this final volume of ethanol, and holding for 1 hr at -70°C. The DNA was removed from the freezer and thawed at room temperature for 15 minutes to insure that no CsCl would precipitate. After 20 minutes of centrifugation at 8,500 rpm in a Sorvall SS34 rotor, the supernatant fraction was discarded and the DNA pellet was dissolved in 0.3 ml 0.3 M sodium acetate. The solution was then transferred to a microcentrifuge tube and 2 volumes of ethanol were added to the tube. This solution was chilled for 5 minutes in -70°C bath, centrifuged for 5 minutes and the supernatant layer was removed. DNA was washed once with 80% cold ethanol, dried in vacuo and resuspended in T.E. buffer. This procedure usually gives a final yield of 1 mg M13 RF DNA per liter of culture.

4. M13 single-stranded DNA preparation.

An overnight culture of JM101 was diluted 1:50 in fresh 2xYT medium and incubated for 2 hrs at 37°C with shaking (200 rpm). One ml of the above culture was diluted again in 50 ml of 2xYT medium. Aliquots (1.6 ml) were dispensed into 10 x 1 cm culture tubes and each was inoculated with material from single well-separated white plaques. The cultures were incubated at 37°C for 4.5 to 6 hrs with shaking (200 rpm) and then transferred to microcentrifuge tubes. The tubes were centrifuged for 5 minutes and the supernatant fraction was transferred to another microcentrifuge tube (1.25 ml). Next, 0.25 ml of 20% PEG 6000 in 2.5 M NaCl was added to the decanted supernatant solution which was mixed well and held for 15 minutes at room temperature. The PEG tubes were

centrifuged for 5 minutes and the supernatant fraction was poured off. The rest of the supernatant fluid was removed with a drawn-out pipet. The tubes were recapped, centrifuged for 10 seconds, and any residual supernatant fluid was completely removed with a drawn-out pipet. The pellet was then resuspended in 10 µl of T.E. (10 mM Tris-HCl, pH 8.0; 0.1 mM EDTA). One hundred µl of a phenol/chloroform mixture (1:1/vol:vol) were added to the solution which was mixed thoroughly for 2 minutes. The tubes were held at room temperature for 5 minutes and agitated with a vortex mixer for another 2 minutes. After 3 minutes of centrifugation, the upper aqueous layer was removed and placed in a new microcentrifuge tube. The DNA was precipitated by the addition of 0.25 ml of a 25:1 mix of ethanol and 3 M sodium acetate, pH 5.5, and holding overnight at -20°C. The DNA was pelleted by centrifugation for 5 minutes, washed once with 70% ethanol (1 ml), dried under vacuum, and resuspended in T.E. buffer.

VIII. Transformation of Escherichia coli.

Competent cells of JM83 were prepared and transformed with pBR322 by the method of Hanahan (35). Competent E. coli JM101 cells were prepared by the following method. One ml of a fresh overnight culture of JM101 was diluted with 100 ml 1xYT medium in a 500 ml flask and incubated until the cells reached an optical density of about 0.5 at 650 nm. The cells were then chilled on ice and pelleted by centrifugation at 6,000 rpm for 6 minutes (Sorvall GSA rotor). The pellet was resuspended in 50 ml of 50 mM $CaCl_2$. The suspension was kept on ice for 20 minutes, pelleted again, and resuspended in 10 ml of 50 mM $CaCl_2$. Host cells of strain JM101 were prepared for plating of M13 phage as

described earlier (see "Large-scale preparation of M13 RF DNA"). Transfection of JM101 by M13 mp9 RF DNA was carried out as outlined below. Glass tubes were chilled on ice and 0.2 ml of JM101-competent cells and 2 µl (4 ng) of ligated DNA were placed into a chilled tube. The tubes were held on ice for 40 minutes and the transfection mixtures were then heat shocked for 2 minutes at 42°C. For each transfection mixture, 15 µl of 100 mM IPTG (isopropylthiogalactoside), 35 µl of 2% X-gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside) in dimethyl formamide and 0.2 ml of JM101 plating culture were added. Three ml of plating agar (soft agar: 0.5% agar solution) held at 45-47°C were added into each tube and mixed completely with gentle agitation on a vortex mixer. The mixture was poured onto a plate at room temperature. The plate was then tilted to obtain an even spreading, covered, cooled for 30 minutes at room temperature, inverted, and incubated at 37°C overnight.

IX. Assay for β-glucosidase.

Cells from broth cultures (1.5 ml:10^8 cells/ml) were pelleted in microcentrifuge tubes and resuspended in 0.5 ml of 0.1 M sodium phosphate buffer, pH 6.5. Ten µl of CTAB (hexadecyl trimethyl ammonium bromide, 2 mg/ml) were added to the suspensions which were incubated at 37°C for 15 minutes. Next, 100 µl of 2 x 10^{-3} M PNPG (P-nitrophenyl β-D-glucopyranoside, Sigma) were added to the tubes which were incubated at 37°C until the appearance of a yellow color.

X. Transposon Mutagenesis.

A single colony of E. coli strain JM83 with pGS2 was inoculated in a culture tube containing 5 ml λym broth and incubated overnight. Fresh

5 ml of λym broth were inoculated with 0.1 ml of above overnight culture and incubated at 37°C with aeration until the cells reached early-log phase (∿5 x 10^8 cells/ml). This corresponded to an OD_{600} of ∿0.2-0.3. Cells were concentrated 10 fold by centrifugation (10 minutes, 10,000 rpm, SS34 rotor) and resuspended in fresh λym broth (0.5 ml final resuspension of 5 ml culture). Four small test tubes were prepared as follows: 1) 0.1 ml cells and 0.1 ml λTn5 (10^9 ϕ/ml, M.O.I. ∿1); 2) 0.1 ml cells and 0.1 ml λTn5 (10^8 ϕ/ml, M.O.I. ∿0.1); 3) 0.1 ml cells; and 4) 0.1 ml λTn5 (same as 1). The tubes were mixed and incubated at room temperature for 30 minutes to allow phage adsorption and infection. To allow for transposition and expression, 1.0 ml of λym broth containing 40 mM sodium citrate was added to tubes and cultures were incubated at 37°C for 90 minutes. Two-tenths ml of the culture was directly spread on selective agar medium containing ampicillin, kanamycin sulfate and 20 mM sodium citrate. Plates were incubated at 37°C overnight (49, 74). Selection for Tn5 inserts in plasmids was carried out as follows. Entire colonies were collected from the plate and the plasmid DNA was isolated by the method of Birnboim and Doly (11). The plasmid DNA was used to transform competent JM83 cells. Individual colonies with Tn5 insertion in plasmid were screened by plating the transformants on selective medium containing kanamycin and ampicillin.

XI. Electrophoresis Techniques.

Horizontal agarose gels were prepared in T.B.E. electrophoresis buffer (89 mM Tris-borate, pH 8.0, containing 2 mM EDTA). Tracking dye (6x) contained 0.25% xylene cyanol, 0.25% bromphenol blue, and 15% ficoll in H_2O. After electrophoresis, gels were stained in a solution

of ethidium bromide (0.5 µg/ml) for 20 to 30 minutes. DNA was made visible on a U.V. transilluminator. HindIII digested λDNA and 123 bp ladder were used as molecular weight markers (purchased from B.R.L.).

For DNA sequencing, 8% polyacrylamide (1x T.B.E.) gels containing 8.3 M urea were used to fractionate ^{35}S-labeled DNA transcripts. Prior to electrophoresis of the samples 40 watts of current were passed through the gel for 30 to 40 minutes. Samples mixed with dye which contained 0.1% bromphenol blue, 0.1% xylene cyanol, and 20 mM EDTA in deionized formamide (FDE) were loaded on the gel. Electrophoresis was carried out at 40 watts for 2 hrs or 4.5 hrs. T.B.E. (1x) was used as the electrophoresis buffer in the upper reservoir and in the lower reservoir. Gels were fixed in a solution containing 10% methanol and 10% glacial acetic acid in water for 15 minutes before transfer to 3 mm paper and drying at 80°C on a BioRad gel dryer. Dried gels were exposed directly to Cronex X-ray film (Dupont) for autoradiography.

XII. Extraction of DNA from Gels.

A slot was cut just ahead of the desired DNA in the gel. A piece of dialysis membrane treated with 2% sodium bicarbonate and 1 mM EDTA was used to line the bottom and distal side of the slot. The slot was filled with T.B.E. buffer and electrophoresis was continued until the DNA had moved against the dialysis membrane. The DNA was pulled off of the membrane by reversing current for 1 to 2 minutes. The buffer containing the DNA was then withdrawn into a microcentrifuge tube and extracted 3 times with butanol. One-tenth volume of 3 M sodium acetate (pH 4.8) and 2 volumes of ethanol of the original DNA volume were added

to the solution. The solution was held at -20°C overnight, and pelleted by centrifugation for 10 minutes. The DNA pellet was washed with 80% ethanol, dried under vacuum and resuspended in T.E. buffer.

Low melting point agarose (B.R.L.) was also used to recover DNA (1% L.M.P. gels were used). Gels were cast and electrophoresed in the normal way except that the agarose was cooled to around 37°C before pouring and solidified for a longer period. After electrophoresis, the gels were stained with a solution of ethidium bromide (0.5 µg/ml) and DNA was made visible with long-wave U.V. The gel band was transferred to a 1.5 ml microcentrifuge tube and heated in a 67-70°C bath until completely melted. An equal volume of T.E. was added to the tube and the mixture was heated another few minutes. The solution was extracted 3 times with an equal volume of phenol saturated with 50 mM Tris-HCl (pH 8.0) and 1 mM EDTA, followed twice by ether extraction and drying at 70°C for 10 minutes . The final volume was measured and transferred to a siliconized microcentrifuge tube. One-tenth volume of 3 M sodium acetate and 2.5 volumes of ethanol were added, mixed, chilled at -70°C for 45 minutes and centrifuged for 10 minutes. The DNA pellet was washed once with 70% ethanol, dried under vacuum, and resuspended in T.E. buffer.

XIII. Nucleic Acid Hybridization Techniques.

DNA was transferred from gels to nitrocellulose membrane (BioRad Transblot Transfer Membrane) by the method of Southern (92).

For dot blots, HindIII digested DNA from the hybrid organism and sonicated DNA from Cellulomonas sp. were denatured at 90 to 95°C for 15 minutes and spotted on nitrocellulose sheets. The sheets were then air dried and baked under vacuum for 2 hrs at 80°C.

Recombinant M13 mp9 derivatives were denatured and DNA was made to adhere to nitrocellulose sheets in the following manner. One µl of M13 lysates was spotted on nitrocellulose sheets and air dried. The sheets were then treated with each of the following solutions for 5 minutes: i) 0.5 M NaOH, ii) 1.5 M NaCl, 0.5 M NaOH, iii) 1.5 M NaCl, 0.5 M Tris-HCl, pH 8.0, and iv) 2x SSPE (SSPE: 0.18 M NaCl, 10 mM NaH_2PO_4, pH 7.4, and 1 mM EDTA, pH 7.4). The sheets were then dried between pieces of 3 mm paper and baked *in vacuo* for 2 hrs at 80°C.

Radioactive probes of circular plasmid were prepared with α-^{32}P-dATP by nick translation according to Rigby *et al.* (76). Radioactive probes of linear DNA were prepared by the following method. A nick translation mixture containing 5 µl of DNA (0.6 µg/µl), 5 µl of 10x nick translation buffer [0.5 M Tris-HCl (pH 7.2), 0.1 M $MgSO_4$, 1 mM di-thiothreitol and 500 µg/ml BSA], dNTP without dATP (1 µl each of 0.2 mM dCTP, dGTP and dTTP), 20 µl of α-^{32}P dATP(5 µCi/µl), 1 µl of DNase I (0.1 µg/µl), 1 µl of DNA polymerase I (10 units/µl) and 17 µl of water was incubated at 15°C for 1 hr. The mixture was then extracted once with phenol. Next, salmon sperm DNA (20 µg) was added to the mixture and nick translated radioactive DNA was co-precipitated with salmon sperm DNA by the addition of 2.5 volumes of ethanol with 0.3 M sodium acetate and holding at -70°C for 30 minutes. The DNA was pelleted by centrifugation at 12,000 rpm for 20 minutes at 0°C (Sigma 2-MK microcentrifuge), washed once with 70% cold ethanol, and dissolved in 100 µl T.E. Buffer. The probe was analyzed by thin layer chromatography (solvent; 0.75 M sodium phosphate buffer, pH 3.5) and radioactivity was

measured with a liquid scintillation spectrometer (Beckman LS2000) (α-^{32}P-deoxyadenosine-5'-triphosphate was purchased from New England Nuclear).

DNA-DNA hybridizations were performed as described in Maniatis, et al. (62) with slight modifications. The baked nitrocellulose sheets to which DNA had been adhered were soaked in 6x SSC (SSC: 0.15 M NaCl, 0.015 M sodium citrate) for 2 minutes. The wet sheets were then placed into heat-sealable bags containing about 50 ml of hybridization buffer (50% formamide, 4x SSC, and 1x Denhardt's solution (17)). Prehybridization was carried out by incubating these bags at 42°C with gentle shaking (100 rpm) for 1 hr. The prehybridization buffer was removed through a cut corner of the bags and fresh hybridization buffer containing 25 µg/ml sonicated, denatured calf thymus carrier DNA was replaced in the bags (200 µl/cm^2). ^{32}P-labeled probes were denatured by heating at 90 to 95°C for 15 minutes and then placing immediately on ice for 5 minutes before addition to the bags. Hybridization was carried out overnight at 42°C with gentle shaking (120 rpm). The hybridization buffer was drained out from the bags. Nitrocellulose sheets were then washed with gentle rocking at room temperature 3 times for 20 minutes with a solution containing 2x SSC and 0.1% SDS, and 2 times for 30 minutes with a solution containing 1x SSC and 0.1% SDS. The sheets were dried at room temperature for 30 minutes, placed on 3 mm paper and covered with saran wrap. Autoradiography was performed by exposing X-ray film with an intensifying screen at -70°C.

XIV. DNA Sequencing Methodology.

Fragments of the presumptive β-glucosidase gene generated by DNase I digestion were cloned into RF DNA of the M13 vector mp9. Transformation of JM101 was performed as described earlier (see "VIII. Transformation of E. coli"). Recombinant phages were screened by nucleic acid hybridization with nick translated linear presumptive β-glucosidase gene. Templates were prepared from positive clones as described earlier. DNA sequencing was then carried out by the methods of Sanger (85) using ddNTPs (P.L. Biochemicals), dNTPs (Boehringer Mannheim), Klenow (Boehringer Mannheim), α-^{35}S-dATP (New England Nuclear) and universal pentadecamer M13 primer (New England Biolabs). Sequencing reactions were analyzed on polyacrylamide gel as described earlier.

Random DNA sequences generated by DNase I were ordered using the computer program of Staden (93). Restriction site, amino acid sequence, and hydropathy analysis of DNA sequence data were carried out using computer programs designed by Douglas Nichol for use with an Apple IIe computer.

RESULTS

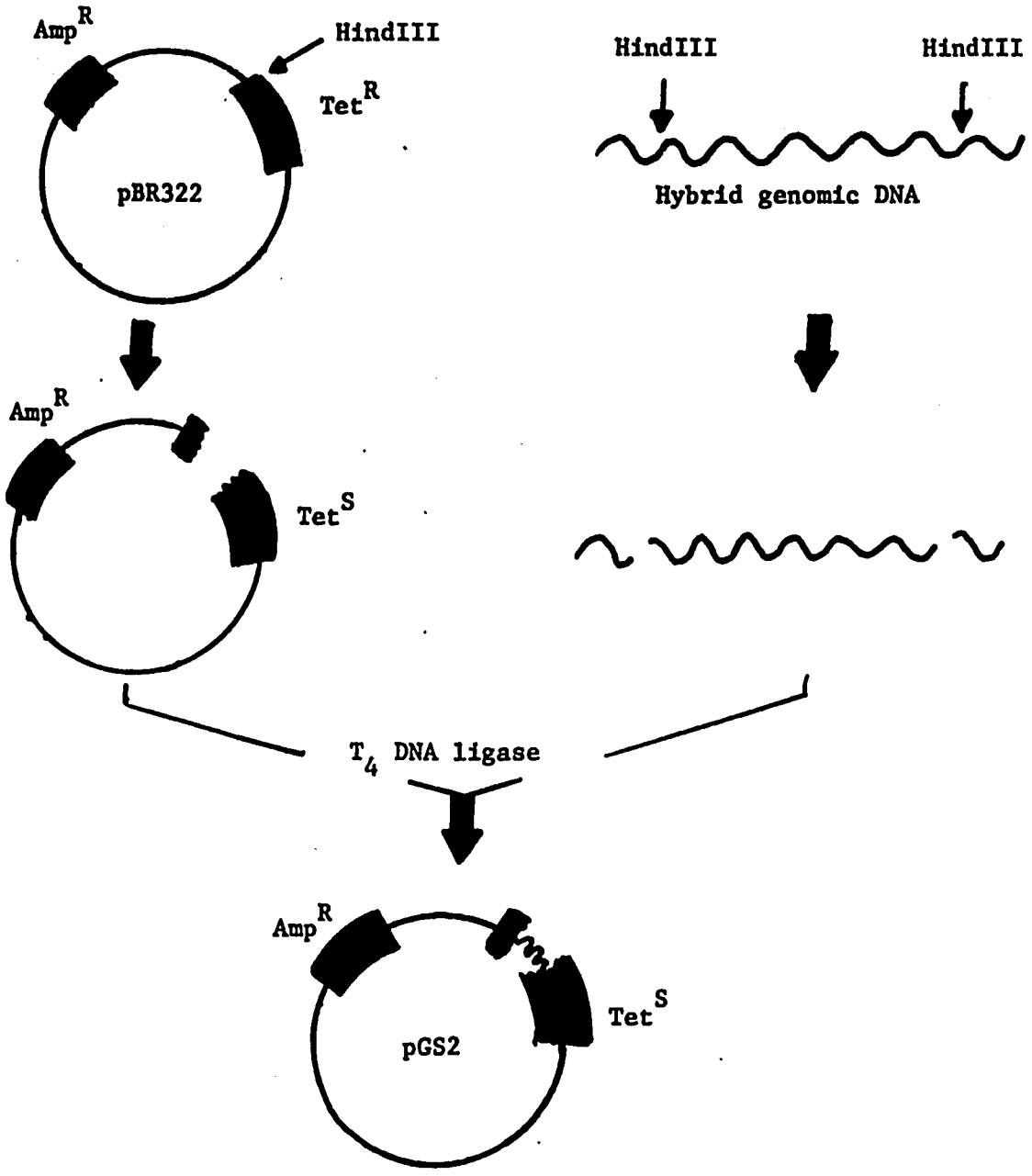
I. Cloning of the β-glucosidase Gene into Escherichia coli Plasmid pBR322.

Initially the genomic DNA of the hybrid bacterium was subjected to partial digestion with the restriction enzyme BamHI to clone a gene encoding a protein which is responsible for β-glucosidase activity into E. coli plasmid pBR322. But it was unsuccessful.

The genomic DNA of the hybrid bacterium was next digested with the restriction enzyme HindIII, and fragments in the range of about 5 to 10 kb were ligated into HindIII digested pBR322 plasmid, and then transformed into E. coli JM83. The methodology for cloning of β-glucosidase gene into E. coli plasmid pBR322 is presented in Figure 3. The transformed cells were selected on L agar medium containing ampicillin. Several thousand colonies appeared after overnight incubation at 37°C. Replica plating of 748 colonies on L agar medium containing tetracycline scored the tetracycline sensitive transformants that likely contained inserts. These transformants (311 colonies), which were sensitive to tetracycline, were tested for growth on minimal cellobiose medium containing ampicillin (40 μg/ml). Since JM83 is unable to grow on cellobiose, transformants growing on the cellobiose medium must be able to hydrolyze the β-1,4-glycosidic bond to release the glucose moiety for use as a carbon source. Four colonies among 311 were found to grow on agar plates with minimal cellobiose medium and ampicillin after 72 hrs incubation at 37°C. Four 10-ml volumes of minimal cellobiose medium containing ampicillin (40 μg/ml) were inoculated with each colony and incubated overnight at 37°C with

Figure 3. The methodology used to clone β-glucosidase gene from a cellulolytic hybrid bacterium into pBR322.

Cloning of the β-glucosidase gene



Selection of Amp^R, Tet^S transformants of E. coli JM83 on cellobiose minimal medium

agitation (250 rpm). Each culture was then plated again on minimal cellobiose medium containing ampicillin and incubated overnight at 37°C. Growth was observed with all 4 clones. These 4 clones were maintained on L agar medium containing ampicillin.

II. Characterization of Presumptive Cellobiose Utilizing Plasmid Clones.

Plasmids were isolated from the four putative clones using the rapid plasmid preparation technique of Birnboim and Doly (11) and then digested with restriction enzyme HindIII. Two of the four putative clones showed insertion of a DNA fragment. These two plasmids were designated as pGS1 and pGS2. Large quantities of pGS1 and pGS2 plasmid DNA were prepared by CsCl equilibrium gradients for further analysis. Figure 4 presents restriction enzyme HindIII digested pGS1, pGS2 and pBR322. The larger fragment was chosen for further study. Since the minimum molecular weight of reported β-glucosidase was around 40,000 daltons, it was felt that the larger fragment might be sufficient to code for the enzyme.

The size of the inserted fragment in pGS2 (1.2 kb) was extrapolated from a standard curve of mobility vs. $\log_{10}$ nucleotide base pairs of HindIII digested λDNA. The standard curve is presented in Figure 5. This result was confirmed later by using a 123 bp DNA fragment ladder of molecular size marker (B.R.L.).

Transformation of E. coli JM83 with purified pGS2 DNA was performed and transformants were selected on minimal cellobiose medium with ampicillin as well as L agar medium with ampicillin. Colonies were counted after overnight incubation. Minimal cellobiose medium with ampicillin had 1.9×10^6 transformants/μg DNA and L agar medium with

Figure 4. Agarose gel electrophoresis of HindIII digests of pBR322 (lane 1), pGS2 (lane 2) and pGS1 (lane 3). HindIII digested λDNA is included as molecular size markers (sizes are kbp).

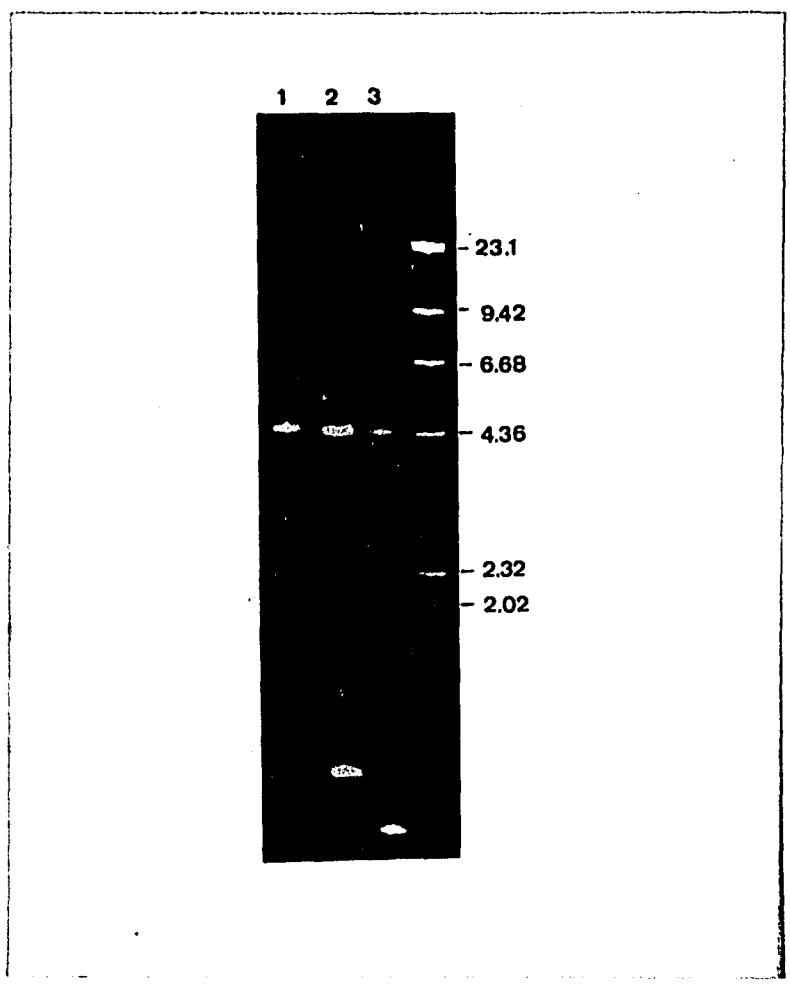
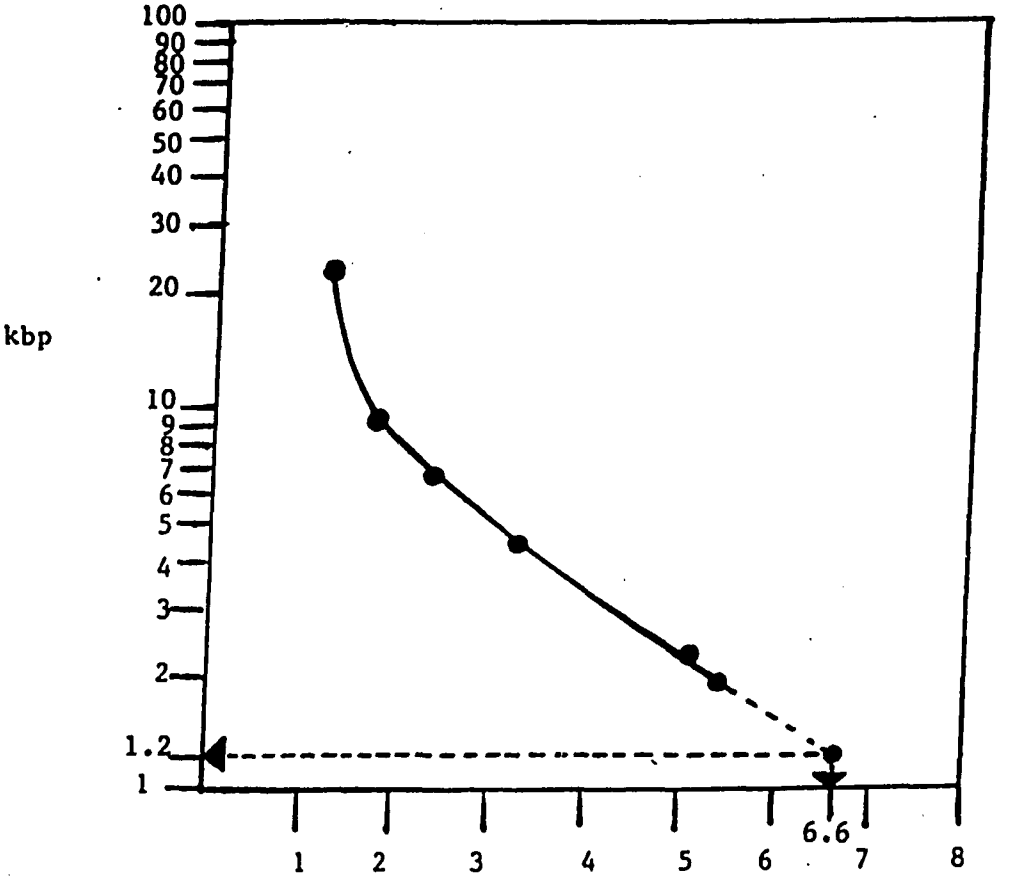

1
2
3
23.1
9.42
6.68
4.36
2.32
2.02

Figure 5. Standard mobility curve ($\log_{10}$ kbp vs. migration in a 0.7% agarose gel) of known DNA fragments used to determine the size of inserted fragment in pGS2.


kbp
100
90
80
70
60
50
40
30
20
10
9
8
7
6
5
4
3
2
1.2
1
1
2
3
4
5
6
6.6
7
8
Distance migrated (cm)

ampicillin had 2.2 x 10^6 transformants/μg DNA. Thus, approximately 85% of transformants on L agar medium containing ampicillin appeared on minimal cellobiose medium with ampicillin. Control transformation with pBR322 DNA resulted in no colonies on cellobiose medium but 2.3 x 10^6 transformants/μg DNA on L agar with ampicillin. Modified MacConkey agar medium which contained cellobiose instead of lactose was also used to detect the ability of transformants to hydrolyze β-1,4-glycosidic linkage. When E. coli JM83, JM83 containing pBR322 and JM83 with pGS2 were plated on modified MacConkey agar medium containing cellobiose, only JM83 with PGS2 showed a color change to red indicating fermentation of added carbohydrate. JM83 and JM83 with pBR322 grew as white colonies on this medium. β-glucosidase activity was tested with chromogenic substrate PNPG as described in materials and methods. Fifteen transformants of pGS2 were selected and grown in broth medium overnight. To 1.5 ml of each culture, PNPG was added. After 24 hrs incubation, hydrolysis of PNPG was confirmed by a color change to yellow which demonstrated that PNPG could be hydrolyzed. When E. coli strains JM83 and JM83 containing pBR322 were incubated with PNPG, there was no color change after 24 hrs showing thereby that PNPG was not hydrolyzed.

III. Transposon Mutagenesis.

λTn5 was used to mutate plasmid pGS2. E. coli strain JM83 containing pGS2 was infected by λTn5 which carried kanamycin resistant gene as described in the materials and methods. The strains carrying Tn5 were selected by L agar medium containing ampicillin and kanamycin. Approximately 1,000 colonies appeared per plate. Plasmids were isolated by the method of Birnboim and Doly (11) and transformed into E coli

strain JM83. The transformants carrying Tn5 in the plasmid were selected by growth on L agar medium containing ampicillin and kanamycin. Twenty plates, each containing about 200-300 colonies, were replicated onto minimal cellobiose medium containing ampicillin and kanamycin. However, no cellobiase negative organisms were detected. Hence, 48 colonies at random were chosen, plasmids were isolated, and examined after HindIII digestion. The 1.2 kb DNA fragment appeared in all clones with one exception. It is probable that the E. coli strain JM83 might have obtained both plasmids - pGS2 and pGS2 with Tn5 - during transformation, or 1.2 kb fragment might be duplicated in the processes of Tn5 mutagenesis. The plasmid which did not have 1.2 kb fragment DNA was used to transform E. coli strain JM83 and then plated on minimal cellobiose medium containing ampicillin and kanamycin and on L agar medium with ampicillin and kanamycin. No growth was observed on minimal cellobiose medium after overnight incubation. Ten colonies at random were chosen from the transformants on L agar plate and examined for their ability to use cellobiose as a carbon source. The organisms did not grow on cellobiose agar after overnight incubation at 37°C. The plasmid was reisolated from the cellobiase negative organism and it was confirmed that it was the same as the one without the 1.2 kb insert. The site of insertion of Tn5 in 1.2 kb fragment was mapped to be 400 bases from 5' end. This estimation was done by the molecular sizes of fragments obtained by digestion of plasmid with restriction enzyme HindIII. This result provided further evidence that the 1.2 kb fragment codes for a protein having β-glucosidase activity.

IV. Southern Transfer and Dot Blot Analyses of Hybrid and Cellulomonas sp. Genomic DNA with pGS2.

Since one of the organisms parental to the hybrid bacterium was a Cellulomonas sp. which is a cellulolytic organism, sequence homology between the cloned gene and Cellulomonas sp. genomic DNA as well as genomic DNA from the hybrid strain was examined by hybridization using Southern transfer and dot blot techniques. Parent Bacillus cereus DNA was not screened because it was presumed that the β-glucosidase gene could be only from Cellulomonas sp. since the original strain of Bacillus cereus did not grow on cellobiose.

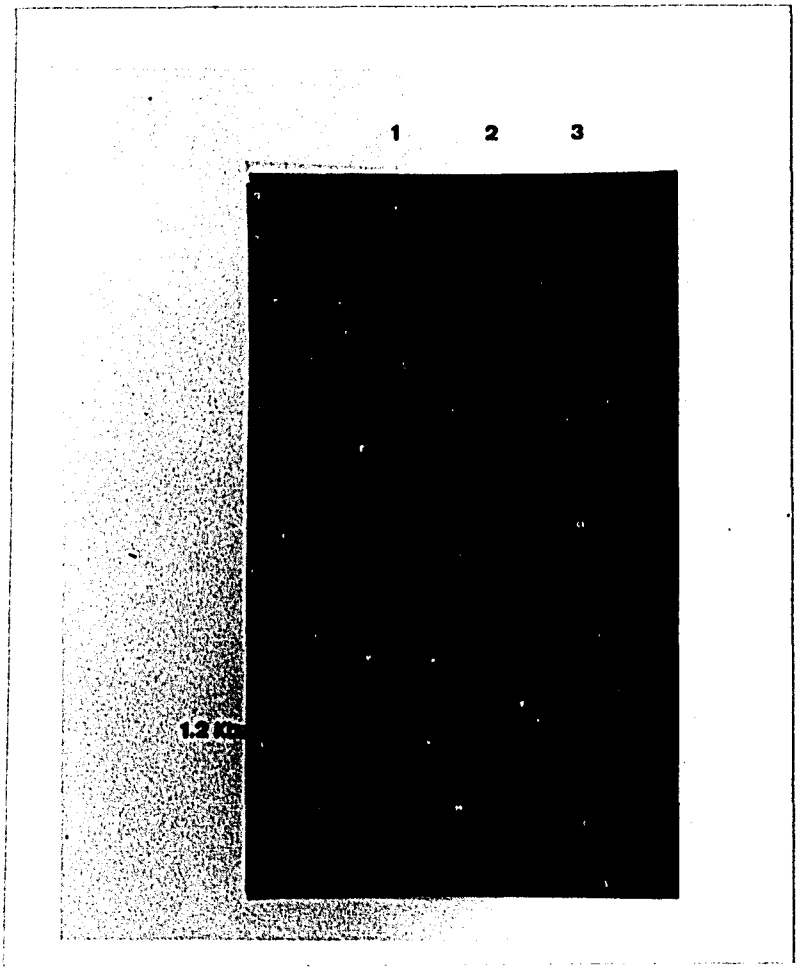
1. Southern transfer analyses.

^{32}P-nick translated pGS2 was used as a probe. DNA from the hybrid bacterium was digested with HindIII. Cellulomonas sp. DNA was double digested with HindIII and BamHI since single digestion with HindIII did not result in proper size fragments. pGS2 was also digested with HindIII. The radiolabeled probe showed hybridization to a single HindIII fragment of DNA from the hybrid bacterium which migrated with the same mobility as the 1.2 kb HindIII fragment encoding the β-glucosidase gene. But no hybridization occurred with Cellulomonas sp. genomic DNA (Figure 6).

2. Dot blot analyses.

Genomic DNA from the hybrid organism was digested with HindIII. Cellulomonas sp. genomic DNA was sonicated (15 seconds sonication with 25 μg DNA in total of 30 μl T.E.) instead of restriction enzyme digestion since double digestion of HindIII and BamHI did not give a proper range of sizes (less than 2 kb). ^{32}P-nick translated pGS2 and

Figure 6. Hybridization of ^{32}P-labeled pGS2 DNA to DNA fragments electrophoretically separated on a 0.7% agarose gel and immobilized on transfer membrane by the method of Southern. 1.2 kb insert from pGS2 (lane 1), HindIII digested DNA from hybrid bacterium (lane 2), and BamHI and HindIII digested DNA from Cellulomonas sp. (lane 3).


1
2
3
1.2

pBR322 were used as probes. pGS2 hybridized to varying amounts of genomic DNA from the hybrid bacterium but not Cellulomonas sp. genomic DNA. pBR322 did not hybridized with either genomic DNA. (The results are not shown.) The results of pGS2 hybridization are presented in Figure 7.

V. Restriction Enzyme Mapping.

Plasmid pGS2 was digested with a variety of endonucleases having a single site in pBR322; furthermore these enzymes recognize specific hexanucleotide sequences. These restriction endonuclease digests were analyzed on 0.7% agarose gel (Figure 8). HindIII digested DNA was used as molecular size markers to construct a standard curve of mobility vs. $\log_{10}$ nucleotide base pair as presented in Figure 9. The data shown in Table 6 (calculated from the standard curve (Figure 9)) were used to determine the restriction map. The restriction map is presented in Figure 10. There were no internal restriction sites for the enzymes AvaI, ClaI, BamHI and SalI (Figure 8). These results were confirmed later by the nucleotide sequence.

VI. Nucleotide Sequence of the Presumptive β-glucosidase Gene.

The dideoxyribonucleotide chain termination method of Sanger et al. (85) was used to determine the nucleotide sequence of the HindIII fragment from pGS2 carrying the β-glucosidase gene. The strategy used to sequence the β-glucosidase gene was as follows. The 1.2 kb fragment was isolated from pGS2 by recovering the HindIII fragment from a 0.7% agarose gel after gel electrophoresis. The HindIII fragments were then ligated in series to form 10 to 20 kb size or bigger DNA with T_4 ligase (Figure 11). Next, these large fragments were subjected to DNase I

Figure 7. Dot blot hybridization of ^{32}P-labeled pGS2 with genomic DNA from the hybrid bacterium (a) and Cellulomonas sp. genomic DNA (b).

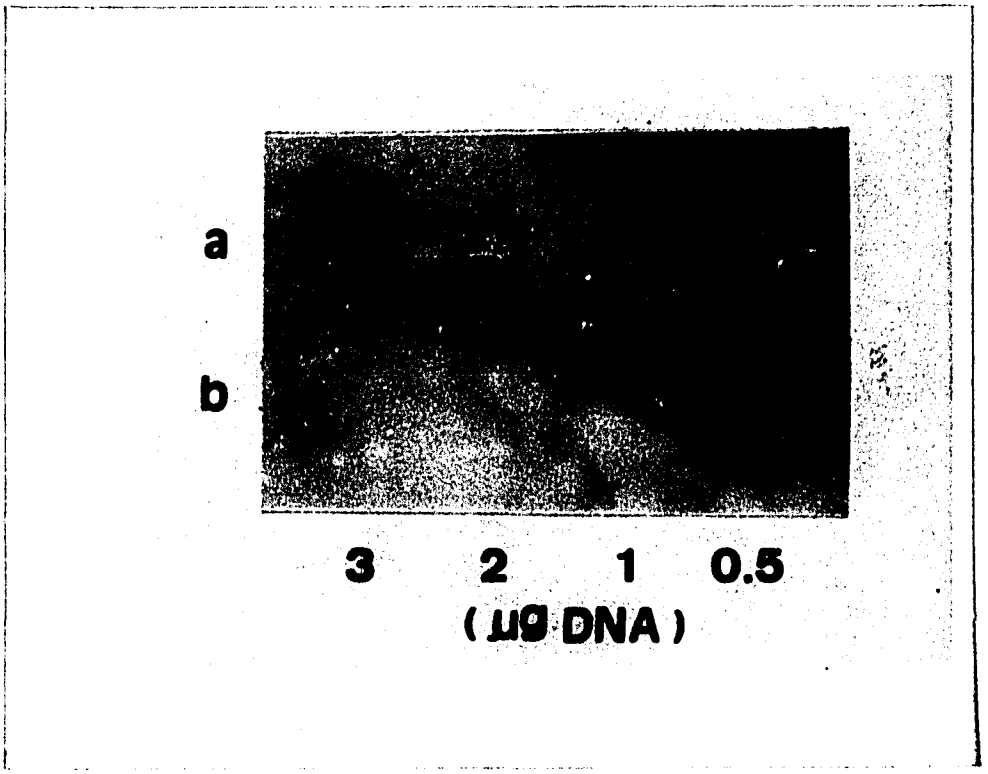

a
b
3
2
1
0.5
(µg DNA)

Figure 8. Agarose gel electrophoresis of restriction enzyme digests of pGS2. pGS2 digested with: PuvII (lane 1), PstI (lane 2), EcoRI (lane 3), BamHI (lane 4), ClaI (lane 5), SalI (lane 6), and AvaI (lane 7). HindIII digested λDNA is included as molecular size markers (sizes are kbp).

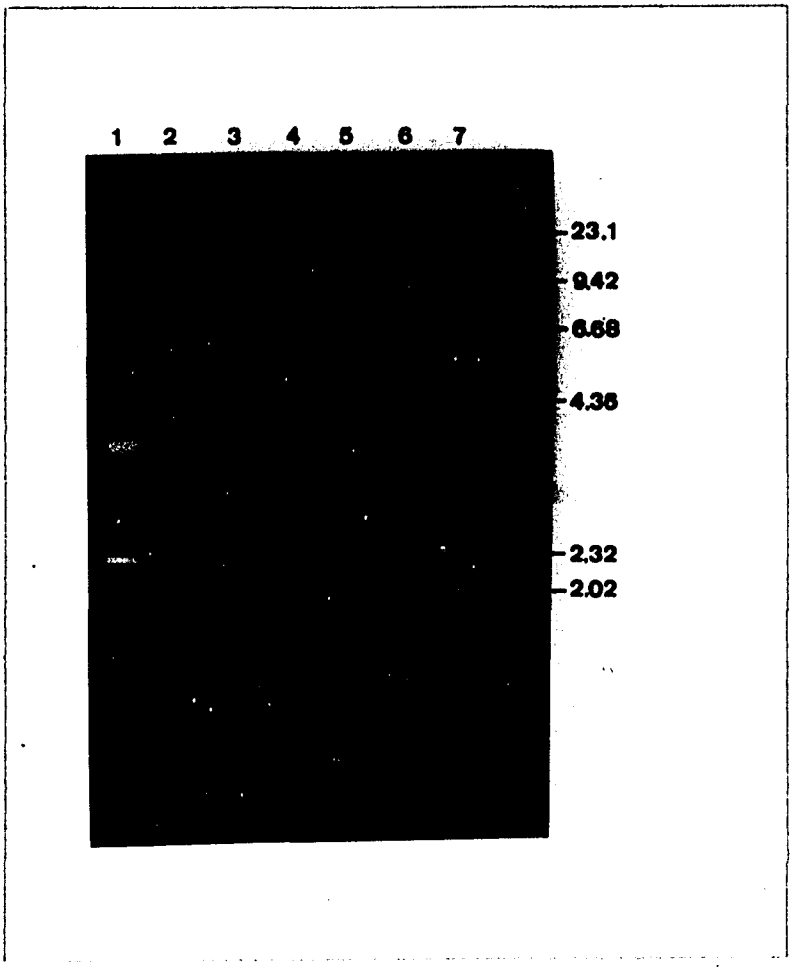

1 2 3 4 5 6 7
23.1
9.42
6.68
4.36
2.32
2.02

Figure 9. Standard mobility curve ($\log_{10}$ kbp vs. migration in a 0.7% agarose gel) of known DNA fragments of data presented in Table 6 which is based on Figure 8 used to determine the size of restriction fragments of pGS2.

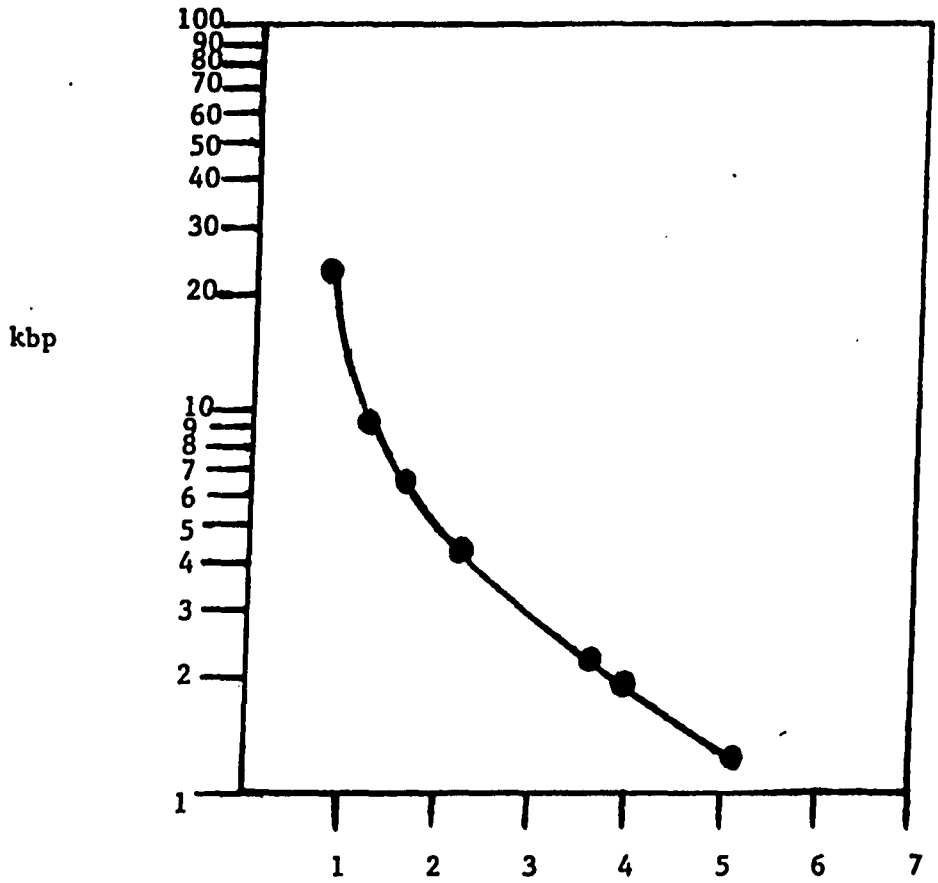

kbp
100
90
80
70
60
50
40
30
20
10
9
8
7
6
5
4
3
2
1
1
2
3
4
5
6
7
Distance migrated (cm)

Table 6. Data for the determination of the restriction map of pGS2.

	Distance migrated (cm)	kbp
HindIII digested λ DNA molecular size marker	0.80	23.130
	1.15	9.416
	1.60	6.682
	2.30	4.361
	3.70	2.322
	4.00	2.207
pGS2 (EcoRI)	2.30	4.360*
	5.10	1.200*
pGS2 (PstI)	2.40	3.900*
	4.30	1.660*
PGS2 (PvuII)	2.55	3.460*
	3.80	2.100*

* calculated from standard curve

Figure 10. Partial restriction enzyme map of pGS2.

Restriction map of pGS2

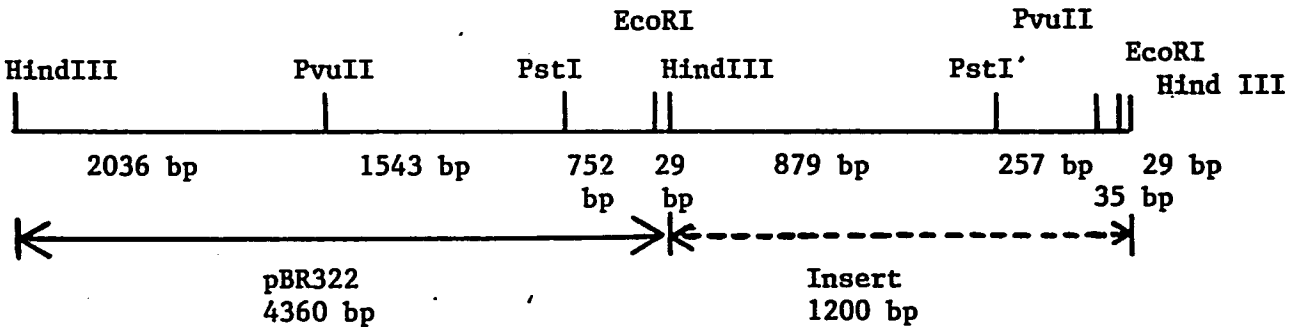
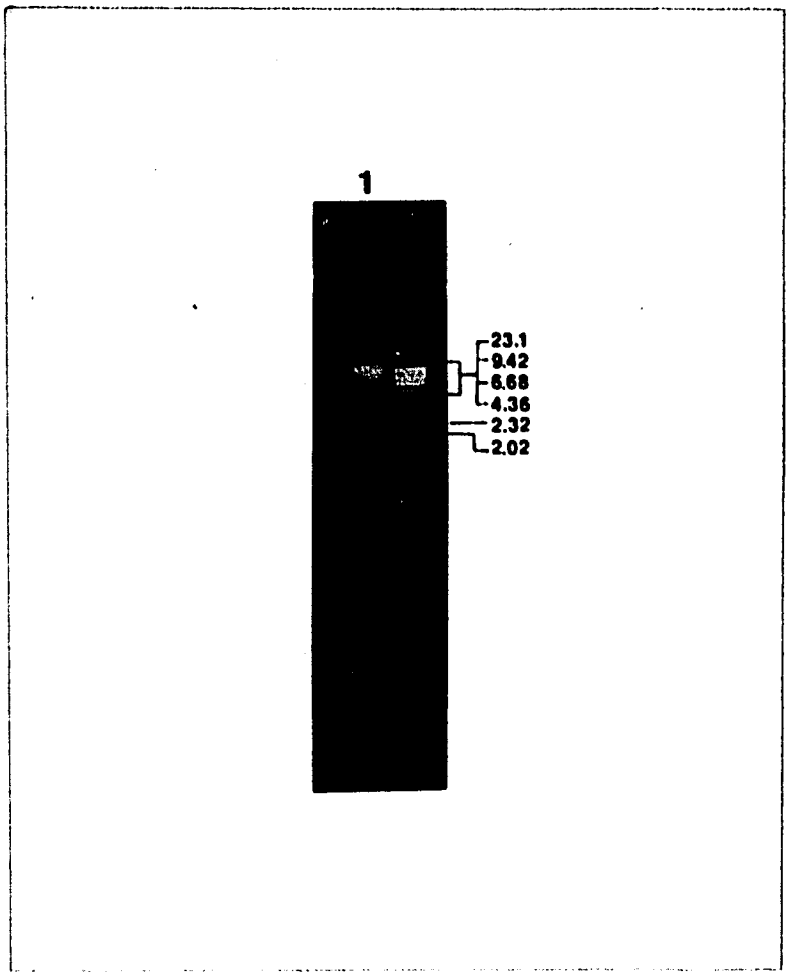


Figure 11. Agarose gel electrophoresis of ligated HindIII fragments of pGS2 (lane 1). HindIII digested λDNA is included as molecular size markers (sizes are kbp).


1
23.1
9.42
6.68
4.36
2.32
2.02

digestion to generate 200-2000 bp size DNA fragments (Figure 12). Since amount of DNase I and time for incubation which generate 200 to 2000 bp size fragments varied according to DNase I prepartion, salmon sperm DNA was digested with DNase I to find the best conditions for the digestion of ligated HindIII fragments. DNase I digested fragments were then repaired and 200 to 1000 bp size fragments were recovered from low melting point agarose gels after gel electrophoresis. These DNA fragments were then cloned into M13 mp9 by the method described earlier. Phage which formed white plaques on JM101 on 1xYT medium in the presence of X-gal contained DNA inserts in the region of the vector encoding the β-galactosidase α-complementing peptide. These white plaques appeared as 5 to 10% of the total phage population. These recombinant phages were screened with ^{32}P-nick translated linear HindIII fragments by dot blot hybridization as shown in Figure 13 (activity of the probe-^{32}P-nick translated 1.2 kb linear HindIII fragments were analyzed by thin layer chromatography (T.L.C.) (Figure 14)). Positive clones were used in sequencing reaction. Also both 5' end DNA strands of HindIII fragment sequence data were obtained by cloning of the entire HindIII fragment into M13 mp9. Figure 15 presents an array of overlapping DNA sequence data generated and ordered by computer analysis (93). The entire HindIII fragment from pGS2 carrying the presumptive β-glucosidase gene was sequenced and is shown in Figure 16.

VII. Analyses of the DNA Sequence of the Presumptive β-glucosidase Gene.

The computer program of Douglas Nichol was used to analyze restriction enzyme sites of the DNA sequence of the HindIII fragment

Figure 12. Electrophoretic pattern of DNase I digested HindIII fragments.

lane 1: 123 bp ladder molecular size markers (sizes are kbp).

lane 2 and 3: DNase I digest of ligated HindIII fragments.

lane 4: HindIII digested λDNA (sizes are kbp).

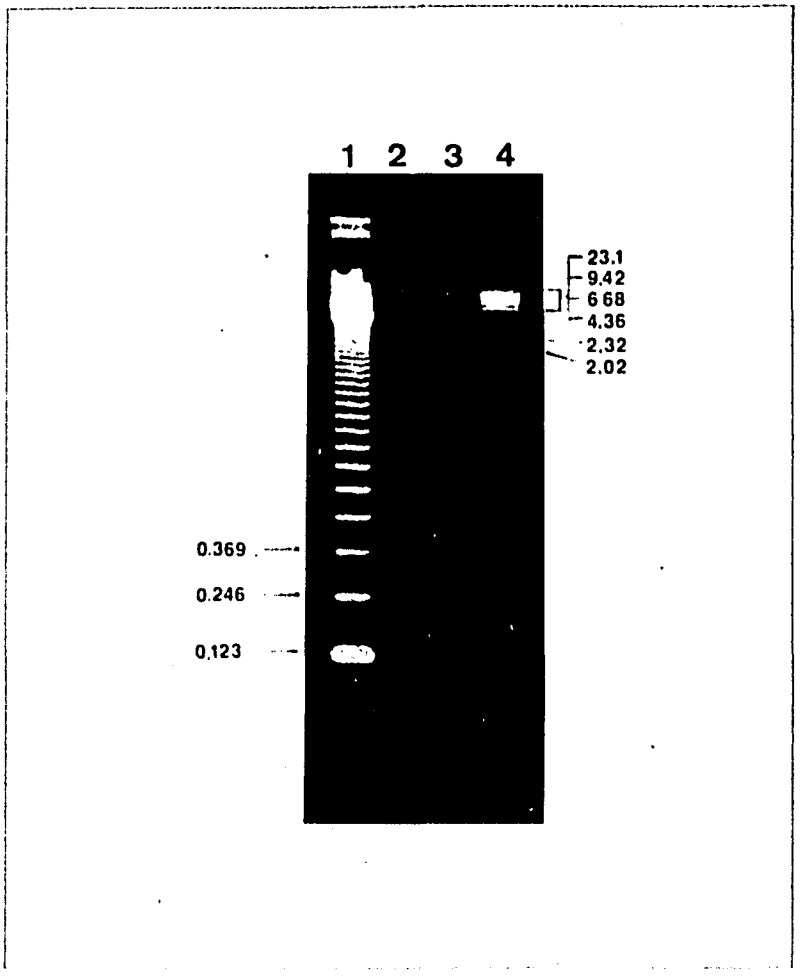

1 2 3 4
23.1
9.42
6.68
4.36
2.32
2.02
0.369
0.246
0.123

Figure 13. Identification of subclones of HindIII fragment in M13 mp9 phage by hybridization with ^{32}P-labeled 1.2 kb HindIII fragments. 105 of 353 recombinants screened for inactivation of β-galactosidase exhibited homology with the probe. M13 mp9 was spotted as a negative hybridization control above the left arrow. 1.2 kb HindIII fragments were spotted as a positive control above the right arrow.

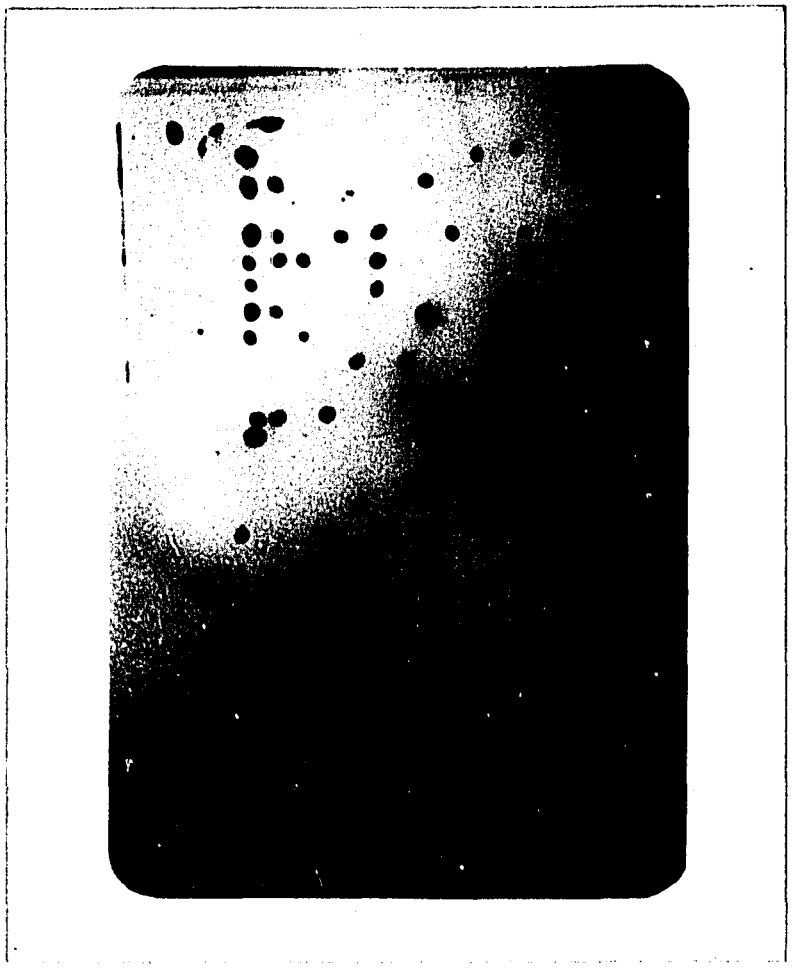


Figure 14. Separation of unincorporated ^{32}P-dATP from nick-translated pGS2 insert DNA using T.L.C. (solvent; sodium acetate buffer, 0.75 M, pH 3.5, development time 30 minutes). Top dot represents unincorporated ^{32}P, bottom dot represents incorporated ^{32}P to HindIII inserts, and middle dot is unknown.

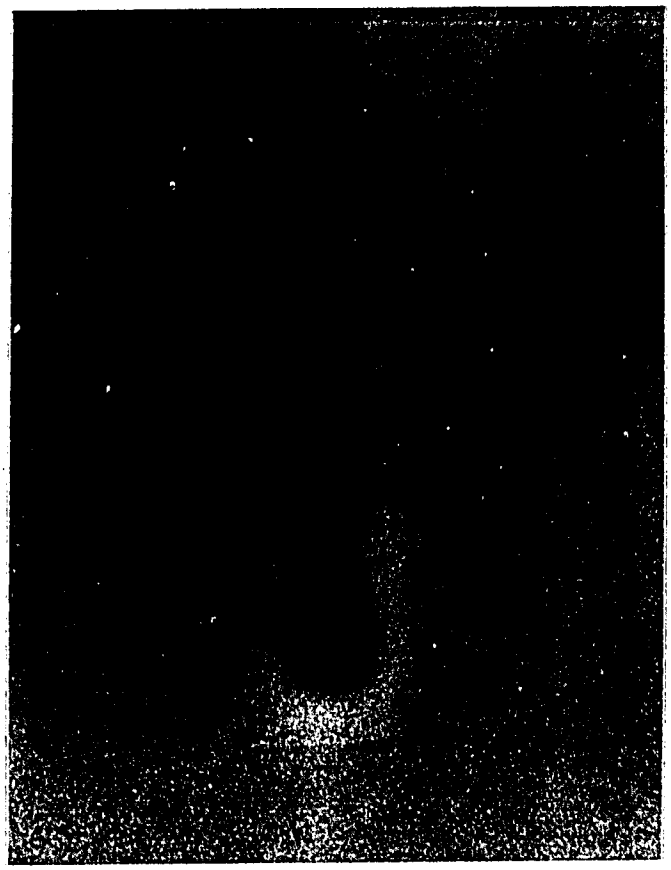
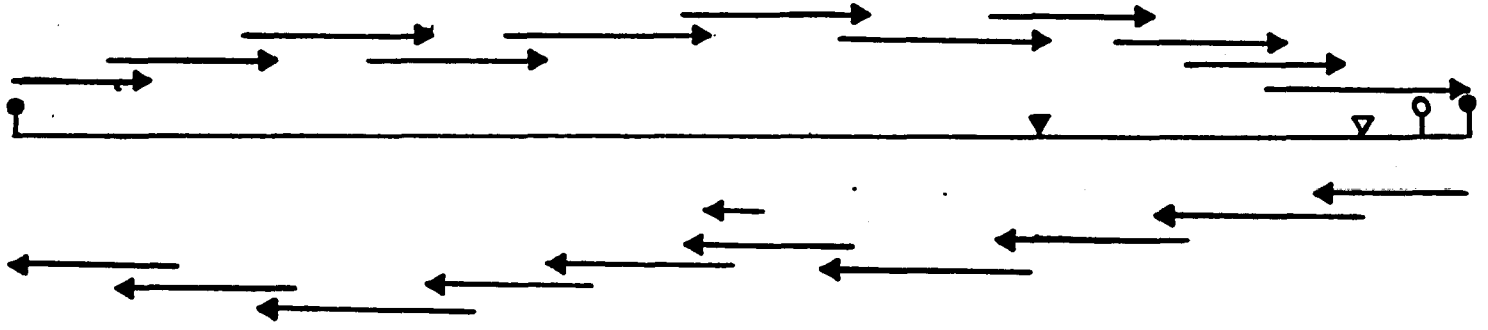


Figure 15. Strategy used to sequence the presumptive β-glucosidase gene.



HindIII

PstI

PvuII

EcoRI

Figure 16. Nucleotide sequence of the HindIII fragment specifying β-glucosidase activity.

box 1: -35 region

box 2: -10 region

box 3: presumptive transcription initiation site

box 4: presumptive translation initiation site

TOTAL NUCLEOTIDES = 1188

```
        10         20         30         40         50         60         70
.........! .........! .........! .........! .........! .........! .........!
AAGCTTGTTC AAAAAGAAAT ACAAGAGCTG GAAGAAACAG CGGCAGAAAA AGCACAAGAA ACCGTTTGTT

        80         90        100        110        120        130        140
.........! .........! .........! .........! .........! .........! .........!
ACAGAAGCAA TATTGCAAAA GAACCAGCTC TTGCAAGAGT TTCGAACGTT GCGACTTCTG TACAGATATT
                                            1                           2
       150        160        170        180        190        200        210
.........! .........! .........! .........! .........! .........! .........!
CTCTGTACTT GCATGGTTGT CGCGATTGTG CTCCAAGTTT GTCGAACGTT CGAAAGACAG GTTCTCCAAA
          3
       220        230        240        250        260        270        280
.........! .........! .........! .........! .........! .........! .........!
AGGAGGTACA TTGTTTATGG TAGGATGCGA TCACGCGAGG ACGAGGCGAA TTTGTACGAC TGTCTGCTTC
                   4
       290        300        310        320        330        340        350
.........! .........! .........! .........! .........! .........! .........!
GACAGCTTTT CTCTTTCTCC TTGCACTAGT TTCGACACCG GATCTCCGTC CATCTTCCAA CAATTTCTGC

       360        370        380        390        400        410        420
.........! .........! .........! .........! .........! .........! .........!
TATTTCTTCT TCCGATAGCA ACAAAAGCTC CGATGTCTAT AAAGACTCGA CATATCGAAG ATACAAATAT

       430        440        450        460        470        480        490
.........! .........! .........! .........! .........! .........! .........!
CGCCGGTTCG ATATCGAACT GGTACAATAT ACCTTTCTTG CACACGAAGA GAGATGTATA TACAGTATCA

       500        510        520        530        540        550        560
.........! .........! .........! .........! .........! .........! .........!
CAGAAACACG AATACTTCGA TGCCGAAGTC TCTGTACAAC ATACGTATAT AGCGAAGAGA CGTACTGGCA

       570        580        590        600        610        620        630
.........! .........! .........! .........! .........! .........! .........!
GAAGTACGAG CAGGGAACCT GGATCGAGTC TTTGGACGAG ACGGAGAAAT TCGCCGCGTT TTCCGCATTC

       640        650        660        670        680        690        700
.........! .........! .........! .........! .........! .........! .........!
TATCGCGAAA AAATATATTG TTCCAGTTTT GCTTCGGGTT CGTCGTCTGC CAGTCGTGGT CTATACCTGC

       710        720        730        740        750        760        770
.........! .........! .........! .........! .........! .........! .........!
TGTCTTGCAT TATACCATAT CCAAAGAGAT GGTTTTATAT TTGTATCCCT TGGCATATCT TTGTCTATGC

       780        790        800        810        820        830        840
.........! .........! .........! .........! .........! .........! .........!
GCTGTGCTAG ACTGGAGCTT CGTGGTACGT GGTTTGCAAG AACGATTTGC AATGGAATAC GGTGTATATA

       850        860        870        880        890        900        910
.........! .........! .........! .........! .........! .........! .........!
CGTACGGGTA CGTCGAACTG CAGCCTTGCT TTGCGTAATC TTTGGAGATA CCTGTTTCTG TGTGTTTGGT

       920        930        940        950        960        970        980
.........! .........! .........! .........! .........! .........! .........!
ATCGTCTTGC ACAGCGCATT GTTCGTCGAG ATGTGCCAGA GGGAATATCT ACGATACGGG AGGGCTTTCT

       990       1000       1010       1020       1030       1040       1050
.........! .........! .........! .........! .........! .........! .........!
TCTGGCCCAG TGCTGCAATG ATACGGAGAC CACGGTACGT CGGATCGGTC CGACATCCGA TCGCTTTTTT

      1060       1070       1080       1090       1100       1110       1120
.........! .........! .........! .........! .........! .........! .........!
CCAGATACAG CAATCGAAAT ATTTGTTGCA GCATGCGCAA CAATTCGAAC AGAAATTGAT ACAGCTGCCA

      1130       1140       1150       1160       1170       1180       1190
.........! .........! .........! .........! .........! .........! .........!
ACAGACACAG ATGAGTACGG AGTGTCTTGG AGCAGAATTC GAGAGCGGAC AGTTGTTCTT CGAAGCTT
```

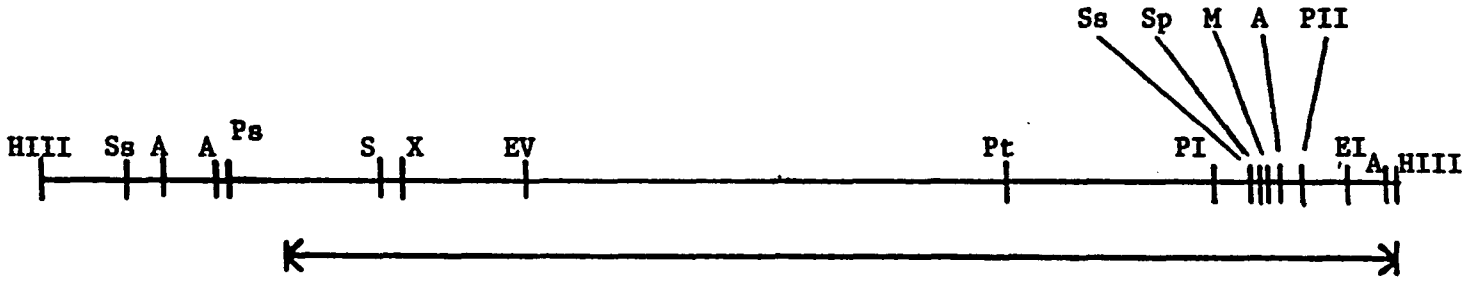
carrying the β-glucosidase gene. Figure 17 presents a map of a few endonuclease sites in the β-glucosidase gene (endonucleases recognize hexanucleotide sequences; obtained from computer search). The restriction sites for PstI, PvuII and EcoRI idendified by enzymatic digestion of HindIII fragments were confirmed by the DNA sequence data. Also, DNA sequence recognition sites for the enzymes (AvaI, ClaI, SalI, and BamHI) which failed to cut the β-glucosidase gene were not present in this DNA sequence.

Translational analysis (Figure 18) of the DNA sequence identified about 12 short open reading frames and 2 incomplete open reading frames. The largest one possibly for β-glucosidase was an incomplete open reading frame beginning with an ATG start codon at position 227. No stop codon was found in this reading frame within the cloned DNA. This open reading frame corresponds to a polypeptide of 319 amino acids approximately 38,000 daltons. Furthermore, the DNA sequence at -18 from the ATG start codon is AAAGGAGG. This probably constitutes the most frequently occurring Shine-Delgarno ribosome binding site in E. coli (23).

A Pribnow box-like region (-10 region; TATTCT at position 137) was observed upstream of the presumptive translational start site (approximately -90 from the initiation codon is present) and possible -35 region (TTCGAA) at position 111 was also observed.

Figure 19 shows computer evaluation of the hydrophilicity and hydrophobicity of the 319 amino acids coding for β-glucosidase. Hydropathy index of the presumptive β-glucosidase gene product was calculated according to Kyte and Doolittle (50) as well as Hopp and

Figure 17. A few selected hexanucleotide sequences recognized by restriction endonuclease identified from DNA sequence of the HindIII fragment specifying β-glucosidase activity (obtained from computer search).



Proposed β-glucosidase gene

Enzyme:	A	: AsuII		Sp	: SphI
	Ps	: Psp 1286		Ss	: SspI
	EV	: EcoRV		X	: XhoII
	PII	: PvuII		HIII	: HindIII
	M	: MstI		EI	: EcoRI
	PI	: PvuI		Pt	: PstI
	S	: SpeI			

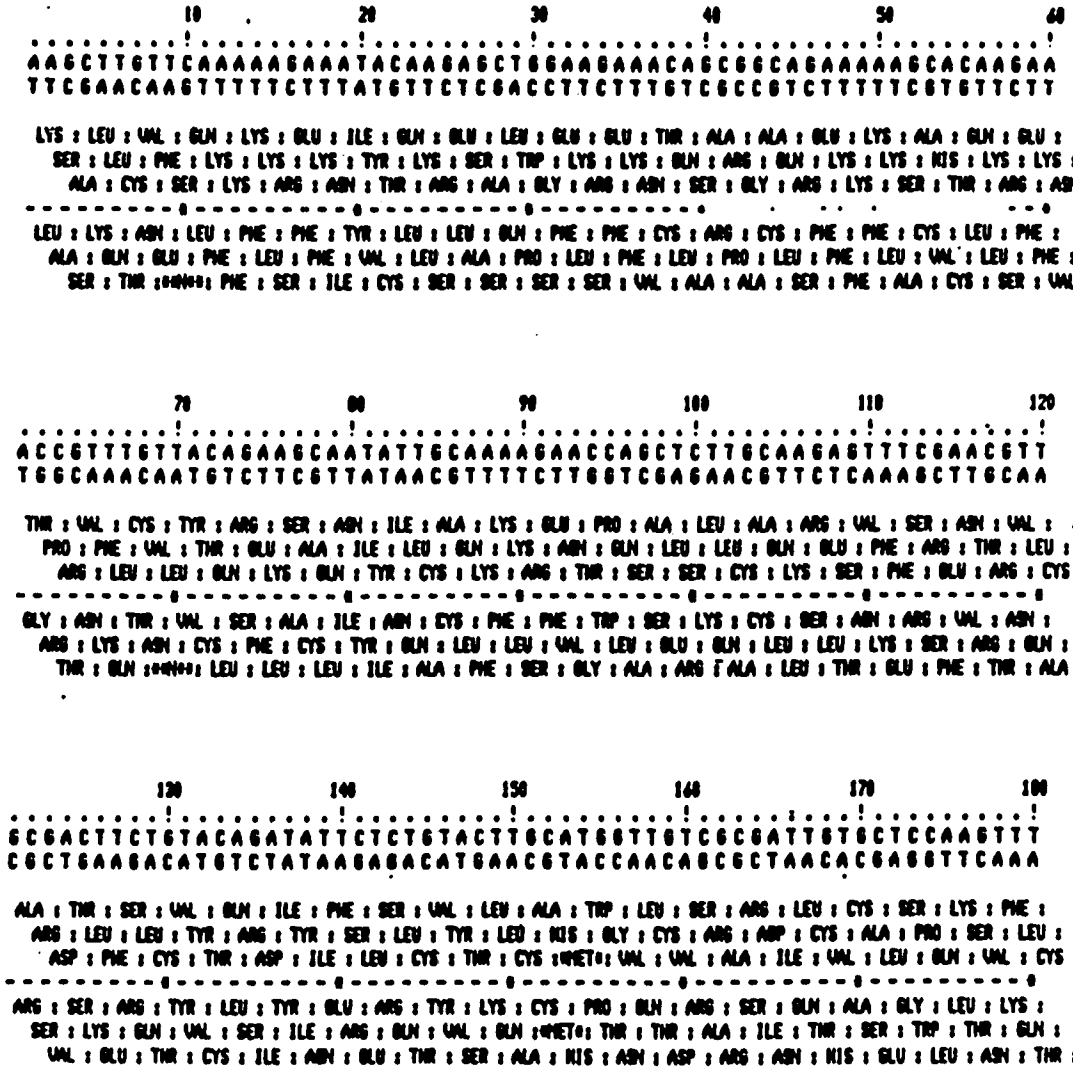
Figure 18. Translational analysis of the sequence of β-glucosidase gene.

box 1: Shine-Delgarno sequence

box 2: presumptive start site

(presumptive amino acid sequences of β-glucosidase gene is underlined).

LENGTH =1188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AAGCTTGTTCAAAAAGAAATACAAGAGCTGGAAGAAACAGCGGCAGAAAAAGCACAAGAA
TTCGAACAAGTTTTTCTTTATGTTCTCGACCTTCTTTGTCGCCGTCTTTTTCGTGTTCTT
LYS : LEU : VAL : GLN : LYS : GLU : ILE : GLN : GLU : LEU : GLU : GLU : THR : ALA : ALA : GLU : LYS : ALA : GLN : GLU :
SER : LEU : PHE : LYS : LYS : LYS : TYR : LYS : SER : TRP : LYS : LYS : GLN : ARG : GLN : LYS : LYS : HIS : LYS : LYS :
ALA : CYS : SER : LYS : ARG : ASN : THR : ARG : ALA : GLY : ARG : ASN : SER : GLY : ARG : LYS : SER : THR : ARG : ASN :
LEU : LYS : ASN : LEU : PHE : PHE : TYR : LEU : LEU : GLN : PHE : PHE : CYS : ARG : CYS : PHE : PHE : CYS : LEU : PHE :
ALA : GLN : GLU : PHE : LEU : PHE : VAL : LEU : ALA : PRO : LEU : PHE : LEU : PRO : LEU : PHE : LEU : VAL : LEU : PHE :
SER : THR :****: PHE : SER : ILE : CYS : SER : SER : SER : SER : VAL : ALA : ALA : SER : PHE : ALA : CYS : SER : VAL :
70 80 90 100 110 120
ACCGTTTGTTACAGAAGCAATATTGCAAAAGAACCAGCTCTTGCAAGAGTTTCGAACGTT
TGGCAAACAATGTCTTCGTTATAACGTTTTCTTGGTCGAGAACGTTCTCAAAGCTTGCAA
THR : VAL : CYS : TYR : ARG : SER : ASN : ILE : ALA : LYS : GLU : PRO : ALA : LEU : ALA : ARG : VAL : SER : ASN : VAL :
PRO : PHE : VAL : THR : GLU : ALA : ILE : LEU : GLN : LYS : ASN : GLN : LEU : LEU : GLN : GLU : PHE : ARG : THR : LEU :
ARG : LEU : LEU : GLN : LYS : GLN : TYR : CYS : LYS : ARG : THR : SER : SER : CYS : LYS : SER : PHE : GLU : ARG : CYS :
GLY : ASN : THR : VAL : SER : ALA : ILE : ASN : CYS : PHE : PHE : TRP : SER : LYS : CYS : SER : ASN : ARG : VAL : ASN :
ARG : LYS : ASN : CYS : PHE : CYS : TYR : GLN : LEU : LEU : VAL : LEU : GLU : GLN : LEU : LEU : LYS : SER : ARG : GLN :
THR : GLN :****: LEU : LEU : LEU : ILE : ALA : PHE : SER : GLY : ALA : ARG : ALA : LEU : THR : GLU : PHE : THR : ALA :
120 140 150 160 170 180
GCGACTTCTGTACAGATATTCTCTGTACTTGCATGGTTGTCGCGATTGTGCTCCAAGTTT
CGCTGAAGACATGTCTATAAGAGACATGAACGTACCAACAGCGCTAACACGAGGTTCAAA
ALA : THR : SER : VAL : GLN : ILE : PHE : SER : VAL : LEU : ALA : TRP : LEU : SER : ARG : LEU : CYS : SER : LYS : PHE :
ARG : LEU : LEU : TYR : ARG : TYR : SER : LEU : TYR : LEU : HIS : GLY : CYS : ARG : ASP : CYS : ALA : PRO : SER : LEU :
ASP : PHE : CYS : THR : ASP : ILE : LEU : CYS : THR : CYS :MET*: VAL : VAL : ALA : ILE : VAL : LEU : GLN : VAL : CYS :
ARG : SER : ARG : TYR : LEU : TYR : GLU : ARG : TYR : LYS : CYS : PRO : GLN : ARG : SER : GLN : ALA : GLY : LEU : LYS :
SER : LYS : GLN : VAL : SER : ILE : ARG : GLN : VAL : GLN :MET*: THR : THR : ALA : ILE : THR : SER : TRP : THR : GLN :
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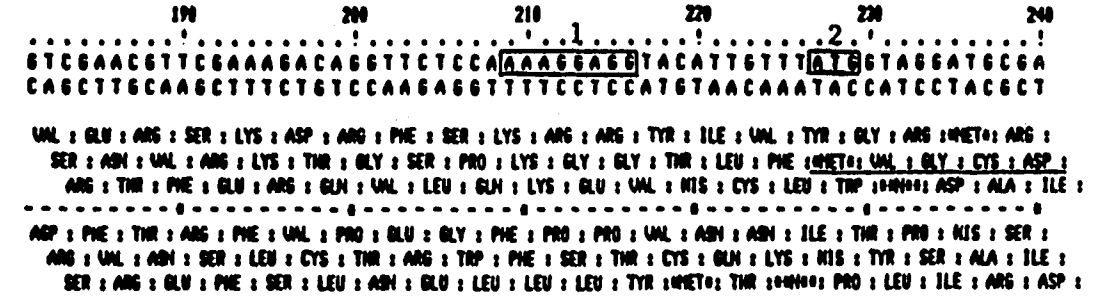
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1
2
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CAGCTTGCAAGCTTTCTGTCCAAGAGGTTTTCCTCCATGTAACAAATACCATCCTACGCT
VAL : GLU : ARG : SER : LYS : ASP : ARG : PHE : SER : LYS : ARG : ARG : TYR : ILE : VAL : TYR : GLY : ARG :
SER : ASN : VAL : ARG : LYS : THR : GLY : SER : PRO : LYS : GLY : GLY : THR : LEU : PHE :
ARG : THR : PHE : GLU : ARG : GLN : VAL : LEU : GLN : LYS : GLU : VAL : HIS : CYS : LEU : TRP :
ASP : PHE : THR : ARG : PHE : VAL : PRO : GLU : GLY : PHE : PRO : PRO : VAL : ASN : ASN : ILE : THR : PRO : HIS : SER :
ARG : VAL : ASN : SER : LEU : CYS : THR : ARG : TRP : PHE : SER : THR : CYS : GLN : LYS : HIS : TYR : SER : ALA : ILE :
SER : ARG : GLU : PHE : SER : LEU : ASN : GLU : LEU : LEU : LEU : TYR :
250 260 270 280 290 300
TCACGCGAGGACGAGCGAATTTGTACGACTGTCTGCTTCGACAGCTTTTCTCTTTCTCC
AGTGCGCTCCTGCTCCGCTTAAACATGCTGACAGACGAAGCTGTCGAAAAGAGAAAGAGG
SER : ARG : GLU : ASP : GLU : ALA : ASN : LEU : TYR : ASP : CYS : LEU : LEU : ARG : GLN : LEU : PHE : SER : PHE : SER :
HIS : ALA : ARG : THR : ARG : ARG : ILE : CYS : THR : THR : VAL : CYS : PHE : ASP : SER : PHE : SER : LEU : SER : PRO :
THR : ARG : GLY : ARG : GLY : GLU : PHE : VAL : ARG : LEU : SER : ALA : SER : THR : ALA : PHE : LEU : PHE : LEU : LEU :
ALA : LEU : VAL : LEU : ARG : ILE : GLN : VAL : VAL : THR : GLN : LYS : SER : LEU : LYS : GLU : ARG : GLU : GLY :
VAL : ARG : PRO : ARG : PRO : SER : ASN : THR : ARG : SER : ASP : ALA : GLU : VAL : ALA : LYS : ARG : LYS : ARG : ARG :
ARG : SER : SER : SER : ALA : PHE : LYS : TYR : SER : GLN : ARG : SER : ARG : CYS : SER : LYS : GLU : LYS : GLU : LYS :
310 320 330 340 350 360
TTGCACTAGTTTCGACACCGGATCTCCGTCCATCTTCCAACAATTTCTGCTATTTCTTCT
AACGTGATCAAAGCTGTGGCCTAGAGGCAGGTAGAAGGTTGTTAAAGACGATAAAGAAGA
LEU : HIS : PHE : ARG : HIS : ARG : ILE : SER : VAL : HIS : LEU : PRO : THR : ILE : SER : ALA : ILE : SER : SER :
CYS : THR : SER : PHE : ASP : THR : GLY : SER : PRO : SER : ILE : PHE : GLN : GLN : PHE : LEU : LEU : PHE : LEU : LEU :
ALA : LEU : VAL : SER : THR : PRO : ASP : LEU : ARG : PRO : SER : SER : ASN : ASN : PHE : CYS : TYR : PHE : PHE : PHE :
GLN : VAL : LEU : LYS : SER : VAL : PRO : ASP : GLY : ASP :
ALA : SER : THR : GLU : VAL : GLY : SER : ARG : ARG : GLY : ASP : GLU : LEU : LEU : LYS : GLN :
CYS :
LYS : TRP : CYS : ASN : ARG : SER : ASN : ARG : ARG :
LYS : LYS : LYS :
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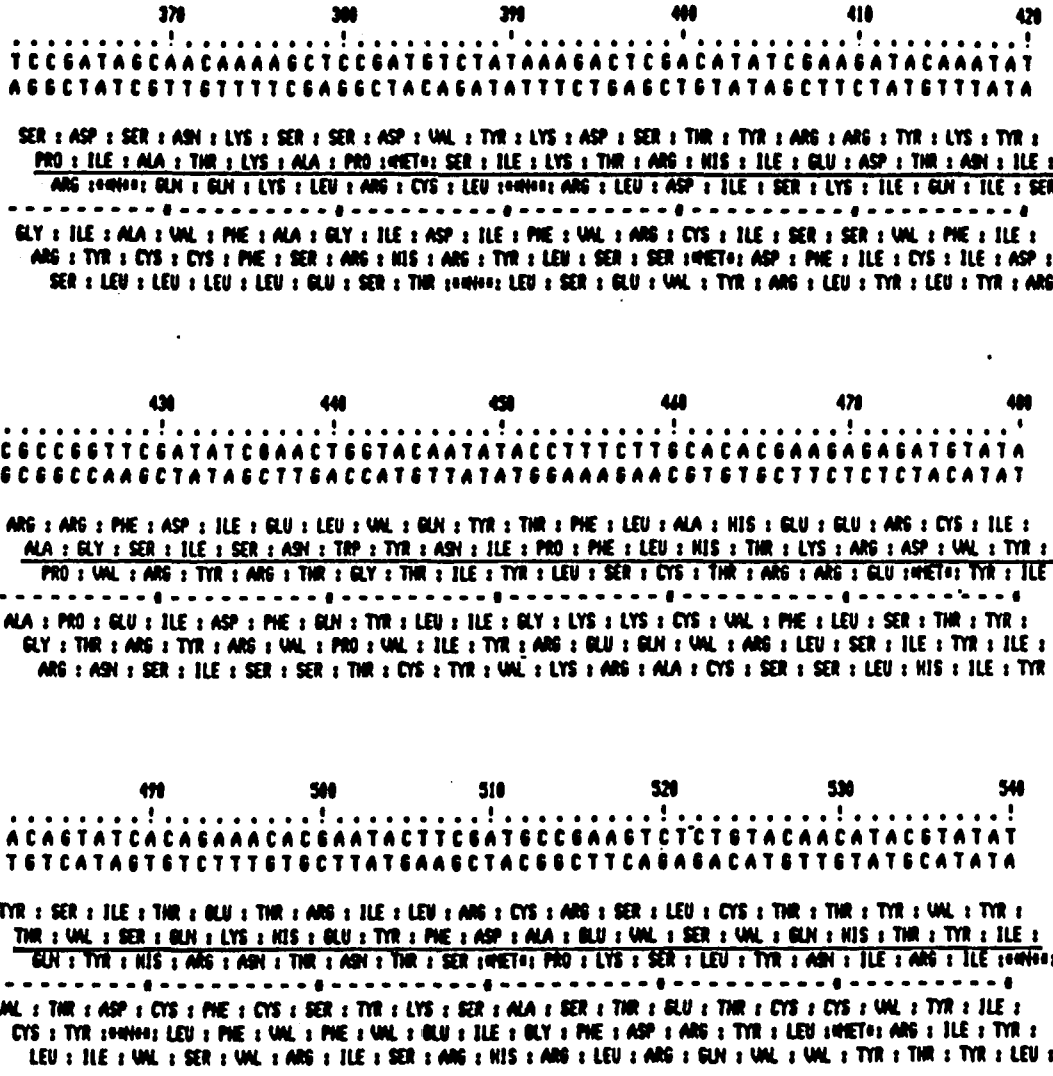
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430 440 450 460 470 480
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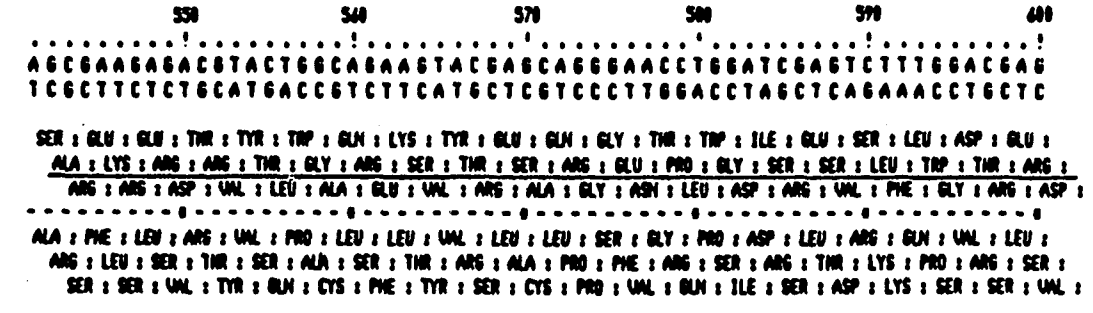
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SER : GLU : GLN : THR : TYR : TRP : GLN : LYS : TYR : GLU : GLN : GLY : THR : TRP : ILE : GLU : SER : LEU : ASP : GLU :
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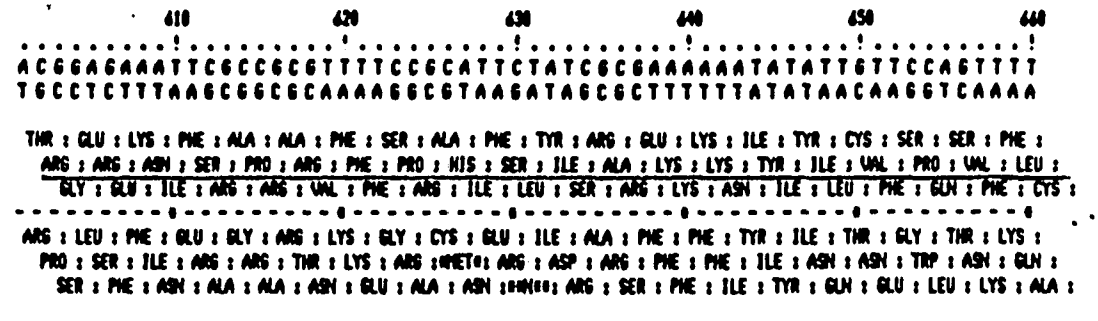
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THR : GLU : LYS : PHE : ALA : ALA : PHE : SER : ALA : PHE : TYR : ARG : GLU : LYS : ILE : TYR : CYS : SER : SER : PHE :
ARG : ARG : ASN : SER : PRO : ARG : PHE : PRO : HIS : SER : ILE : ALA : LYS : LYS : TYR : ILE : VAL : PRO : VAL : LEU :
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PRO : SER : ILE : ARG : ARG : THR : LYS : ARG : MET : ARG : ASP : ARG : PHE : PHE : ILE : ASN : ASN : TRP : ASN : GLN :
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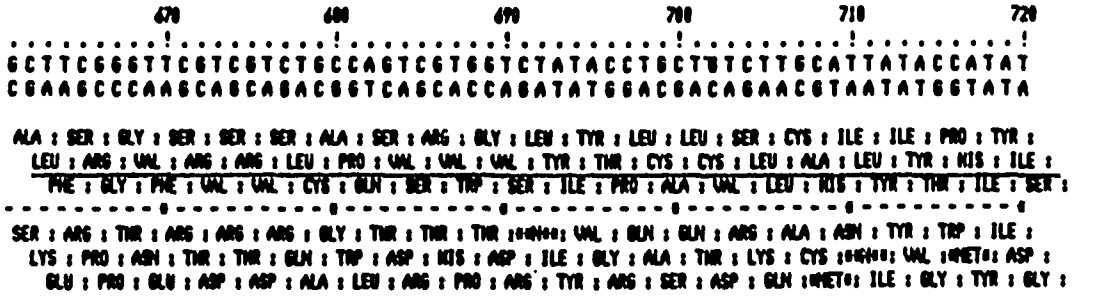
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ALA : SER : GLY : SER : SER : SER : ALA : SER : ARG : GLY : LEU : TYR : LEU : LEU : SER : CYS : ILE : ILE : PRO : TYR :
LEU : ARG : VAL : ARG : ARG : LEU : PRO : VAL : VAL : VAL : TYR : THR : CYS : CYS : LEU : ALA : LEU : TYR : HIS : ILE :
PHE : GLY : PHE : VAL : VAL : CYS : GLN : SER : TRP : SER : ILE : PRO : ALA : VAL : LEU : HIS : TYR : THR : ILE : SER :
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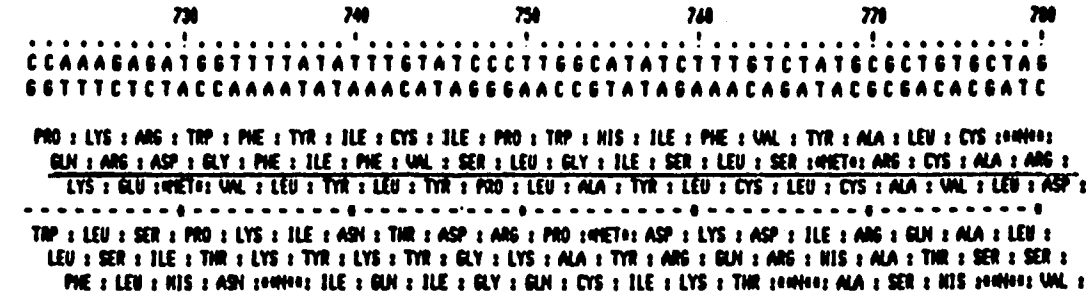
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GLN : ARG : ASP : GLY : PHE : ILE : PHE : VAL : SER : LEU : GLY : ILE : SER : LEU : SER : MET : ARG : CYS : ALA : ARG :
LYS : GLU : MET : VAL : LEU : TYR : LEU : TYR : PRO : LEU : ALA : TYR : LEU : CYS : LEU : CYS : ALA : VAL : LEU : ASP :
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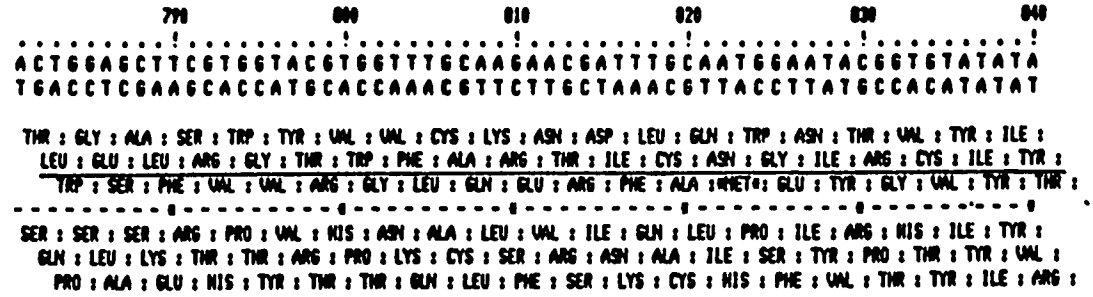
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LEU : GLU : LEU : ARG : GLY : THR : TRP : PHE : ALA : ARG : THR : ILE : CYS : ASN : GLY : ILE : ARG : CYS : ILE : TYR :
TRP : SER : PHE : VAL : VAL : ARG : GLY : LEU : GLN : GLU : ARG : PHE : ALA : MET : GLU : TYR : GLY : VAL : TYR : THR :
SER : SER : SER : ARG : PRO : VAL : HIS : ASN : ALA : LEU : VAL : ILE : GLN : LEU : PRO : ILE : ARG : HIS : ILE : TYR :
GLN : LEU : LYS : THR : THR : ARG : PRO : LYS : CYS : SER : ARG : ASN : ALA : ILE : SER : TYR : PRO : THR : TYR : VAL :
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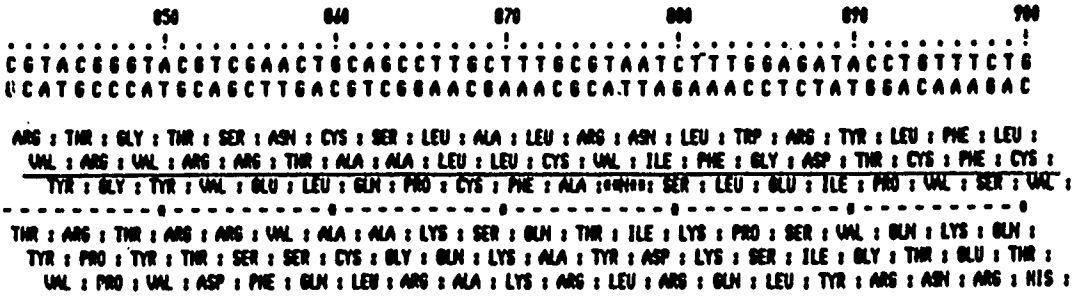
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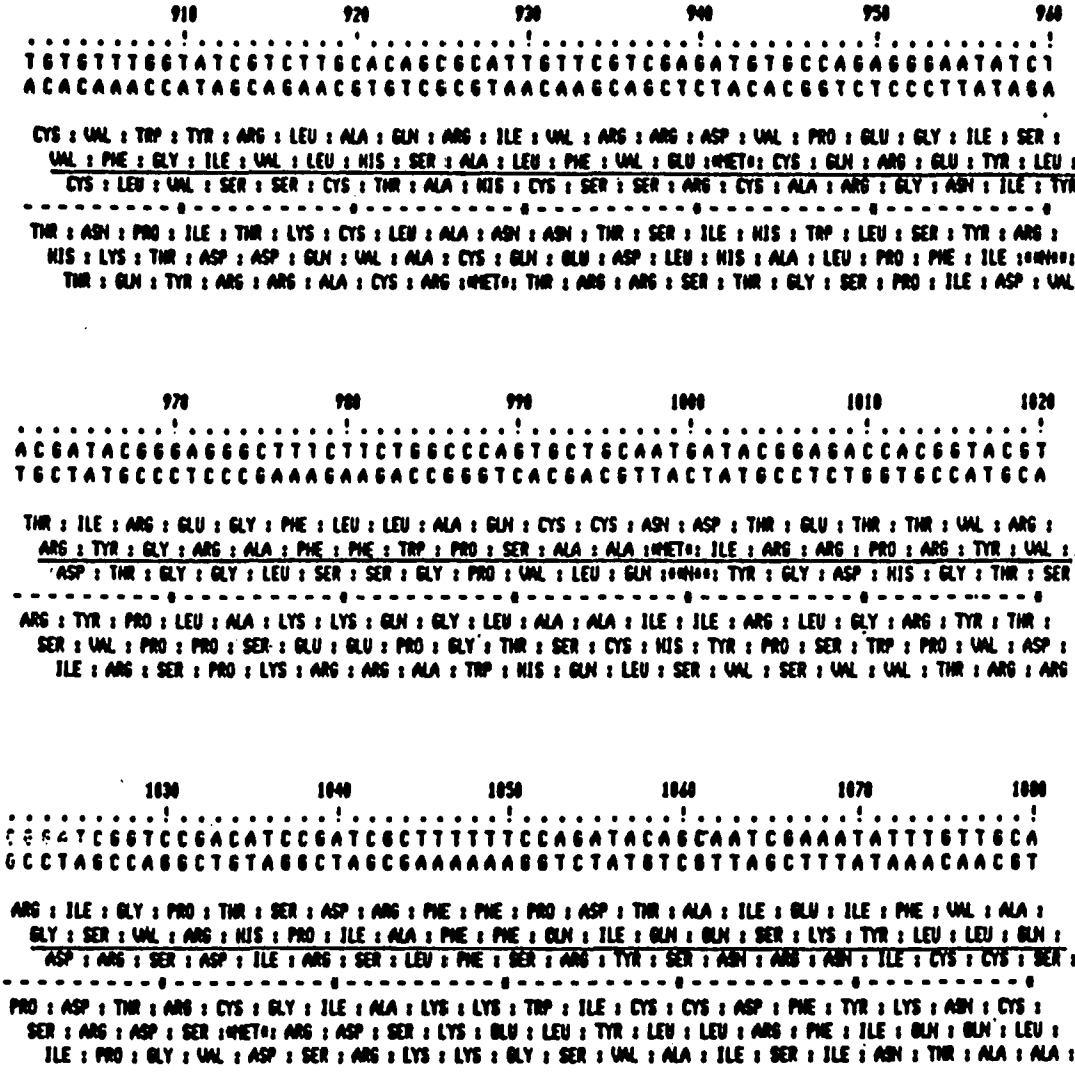
ARG : THR : GLY : THR : SER : ASN : CYS : SER : LEU : ALA : LEU : ARG : ASN : LEU : TRP : ARG : TYR : LEU : PHE : LEU :
VAL : ARG : VAL : ARG : ARG : THR : ALA : ALA : LEU : LEU : CYS : VAL : ILE : PHE : GLY : ASP : THR : CYS : PHE : CYS :
TYR : GLY : TYR : VAL : GLU : LEU : GLN : PRO : CYS : PHE : ALA : : SER : LEU : GLU : ILE : PRO : VAL : SER : VAL :
THR : ARG : THR : ARG : ARG : VAL : ALA : ALA : LYS : SER : GLN : THR : ILE : LYS : PRO : SER : VAL : GLN : LYS : GLN :
TYR : PRO : TYR : THR : SER : SER : CYS : GLY : GLN : LYS : ALA : TYR : ASP : LYS : SER : ILE : GLY : THR : GLU : THR :
VAL : PRO : VAL : ASP : PHE : GLN : LEU : ARG : ALA : LYS : ARG : LEU : ARG : GLN : LEU : TYR : ARG : ASN : ARG : HIS :


910 920 930 940 950 960
TGTGTTTGGTATCGTCTTGCACAGCGCATTGTTCGTCGAGATGTGCCAGAGGGAATATC1
ACACAAACCATAGCAGAACGTGTCGCGTAACAAGCAGCTCTACACGGTCTCCCTTATAGA
CYS : VAL : TRP : TYR : ARG : LEU : ALA : GLN : ARG : ILE : VAL : ARG : ARG : ASP : VAL : PRO : GLU : GLY : ILE : SER :
VAL : PHE : GLY : ILE : VAL : LEU : HIS : SER : ALA : LEU : PHE : VAL : GLU :*MET*: CYS : GLN : ARG : GLU : TYR : LEU :
CYS : LEU : VAL : SER : SER : CYS : THR : ALA : HIS : CYS : SER : SER : ARG : CYS : ALA : ARG : GLY : ASN : ILE : TYR :
THR : ASN : PRO : ILE : THR : LYS : CYS : LEU : ALA : ASN : ASN : THR : SER : ILE : HIS : TRP : LEU : SER : TYR : ARG :
HIS : LYS : THR : ASP : ASP : GLN : VAL : ALA : CYS : GLN : GLU : ASP : LEU : HIS : ALA : LEU : PRO : PHE : ILE :*HIS*:
THR : GLN : TYR : ARG : ARG : ALA : CYS : ARG :*MET*: THR : ARG : ARG : SER : THR : GLY : SER : PRO : ILE : ASP : VAL :

970 980 990 1000 1010 1020
ACGATACGGGAGGGCTTTCTTCTGGCCCAGTGCTGCAATGATACGGAGACCACGGTACGT
TGCTATGCCCTCCCGAAAGAAGACCGGGTCACGACGTTACTATGCCTCTGGTGCCATGCA
THR : ILE : ARG : GLU : GLY : PHE : LEU : LEU : ALA : GLN : CYS : CYS : ASN : ASP : THR : GLU : THR : THR : VAL : ARG :
ARG : TYR : GLY : ARG : ALA : PHE : PHE : TRP : PRO : SER : ALA : ALA :*MET*: ILE : ARG : ARG : PRO : ARG : TYR : VAL :
ASP : THR : GLY : GLY : LEU : SER : SER : GLY : PRO : VAL : LEU : GLN :*HIS*: TYR : GLY : ASP : HIS : GLY : THR : SER :
ARG : TYR : PRO : LEU : ALA : LYS : LYS : GLN : GLY : LEU : ALA : ALA : ILE : ILE : ARG : LEU : GLY : ARG : TYR : THR :
SER : VAL : PRO : PRO : SER : GLU : GLU : PRO : GLY : THR : SER : CYS : HIS : TYR : PRO : SER : TRP : PRO : VAL : ASP :
ILE : ARG : SER : PRO : LYS : ARG : ARG : ALA : TRP : HIS : GLN : LEU : SER : VAL : SER : VAL : VAL : THR : ARG : ARG :

1030 1040 1050 1060 1070 1080
CCGATCGGTCCGACATCCGATCGCTTTTTTCCAGATACAGCAATCGAAATATTTGTTGCA
GCCTAGCCAGGCTGTAGGCTAGCGAAAAAAGGTCTATGTCGTTAGCTTTATAAACAACGT
ARG : ILE : GLY : PRO : THR : SER : ASP : ARG : PHE : PHE : PRO : ASP : THR : ALA : ILE : GLU : ILE : PHE : VAL : ALA :
GLY : SER : VAL : ARG : HIS : PRO : ILE : ALA : PHE : PHE : GLN : ILE : GLN : GLN : SER : LYS : TYR : LEU : LEU : GLN :
ASP : ARG : SER : ASP : ILE : ARG : SER : LEU : PHE : SER : ARG : TYR : SER : ASN : ARG : ASN : ILE : CYS : CYS : SER :
PRO : ASP : THR : ARG : CYS : GLY : ILE : ALA : LYS : LYS : TRP : ILE : CYS : CYS : ASP : PHE : TYR : LYS : ASN : CYS :
SER : ARG : ASP : SER :*MET*: ARG : ASP : SER : LYS : GLU : LEU : TYR : LEU : LEU : ARG : PHE : ILE : GLN : GLN : LEU :
ILE : PRO : GLY : VAL : ASP : SER : ARG : LYS : LYS : GLY : SER : VAL : ALA : ILE : SER : ILE : ASN : THR : ALA : ALA :

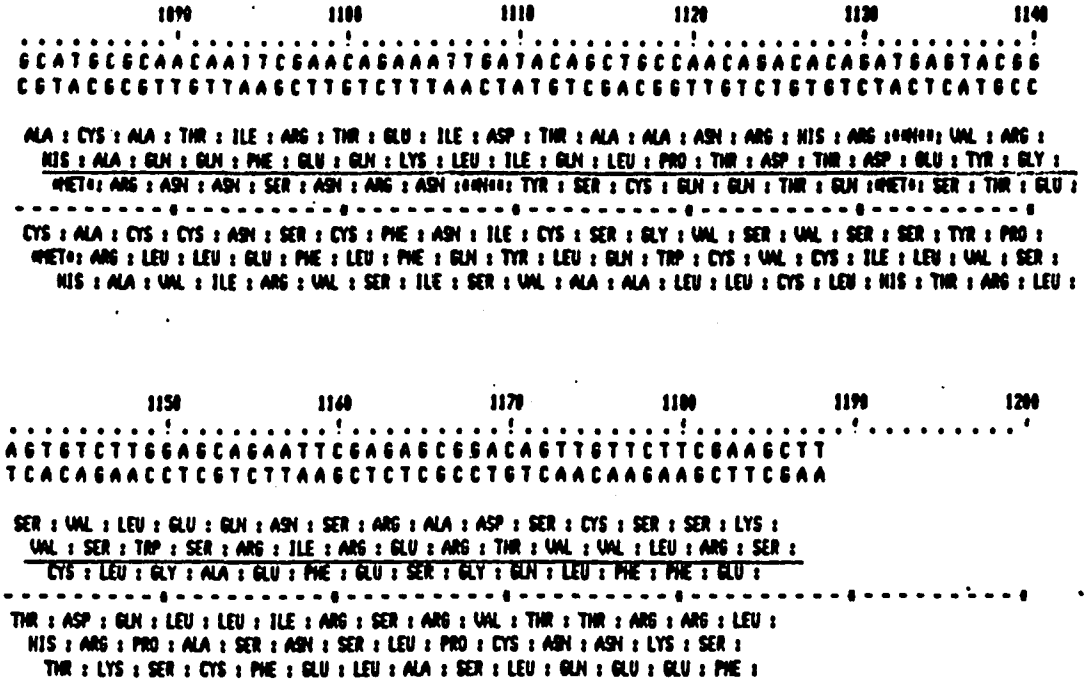

1090 1100 1110 1120 1130 1140
GCATGCGCAACAATTCGAACAGAAATTGATACAGCTGCCAACAGACACAGATGAGTACGG
CGTACGCGTTGTTAAGCTTGTCTTTAACTATGTCGACGGTTGTCTGTGTCTACTCATGCC
ALA : CYS : ALA : THR : ILE : ARG : THR : GLU : ILE : ASP : THR : ALA : ALA : ASN : ARG : HIS : ARG :***: VAL : ARG :
HIS : ALA : GLN : GLN : PHE : GLU : GLN : LYS : LEU : ILE : GLN : LEU : PRO : THR : ASP : THR : ASP : GLU : TYR : GLY :
MET: ARG : ASN : ASN : SER : ASN : ARG : ASN :***: TYR : SER : CYS : GLN : GLN : THR : GLN :*MET*: SER : THR : GLU :
CYS : ALA : CYS : CYS : ASN : SER : CYS : PHE : ASN : ILE : CYS : SER : GLY : VAL : SER : VAL : SER : SER : TYR : PRO :
MET: ARG : LEU : LEU : GLU : PHE : LEU : PHE : GLN : TYR : LEU : GLN : TRP : CYS : VAL : CYS : ILE : LEU : VAL : SER :
HIS : ALA : VAL : ILE : ARG : VAL : SER : ILE : SER : VAL : ALA : ALA : LEU : LEU : CYS : LEU : HIS : THR : ARG : LEU :
1150 1160 1170 1180 1190 1200
AGTGTCTTGGAGCAGAATTCGAGAGCGGACAGTTGTTCTTCGAAGCTT
TCACAGAACCTCGTCTTAAGCTCTCGCCTGTCAACAAGAAGCTTCGAA
SER : VAL : LEU : GLU : GLN : ASN : SER : ARG : ALA : ASP : SER : CYS : SER : SER : LYS :
VAL : SER : TRP : SER : ARG : ILE : ARG : GLU : ARG : THR : VAL : VAL : LEU : ARG : SER :
CYS : LEU : GLY : ALA : GLU : PHE : GLU : SER : GLY : GLN : LEU : PHE : PHE : GLU :
THR : ASP : GLN : LEU : LEU : ILE : ARG : SER : ARG : VAL : THR : THR : ARG : ARG : LEU :
HIS : ARG : PRO : ALA : SER : ASN : SER : LEU : PRO : CYS : ASN : ASN : LYS : SER :
THR : LYS : SER : CYS : PHE : GLU : LEU : ALA : SER : LEU : GLN : GLU : GLU : PHE :

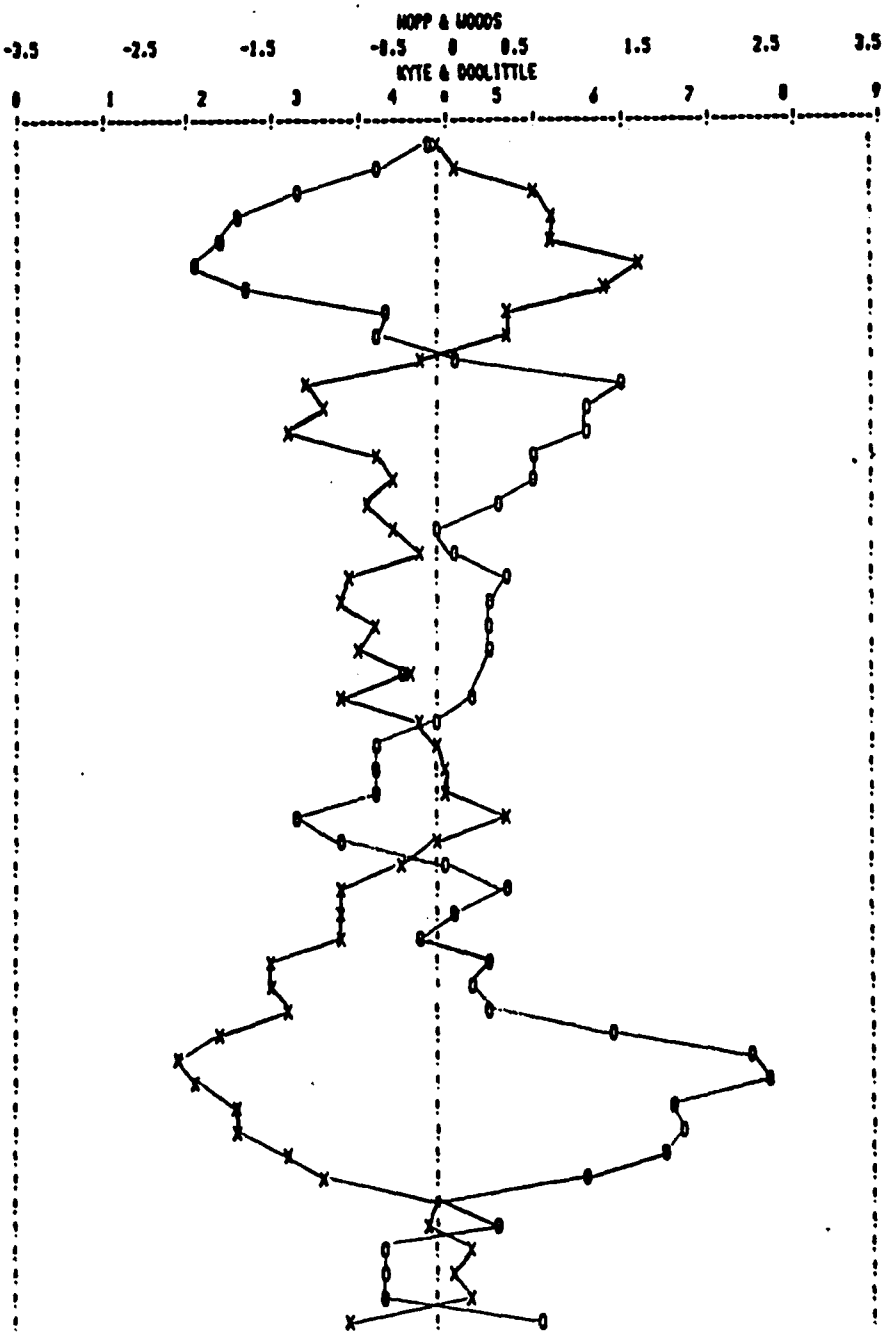
Figure 19. Hydropathy analysis of the β-glucosidase gene nucleotide sequence.

X — HOPP & WOODS PARAMETER

O — KYTE & DOOLITTLE'S PARAMETER

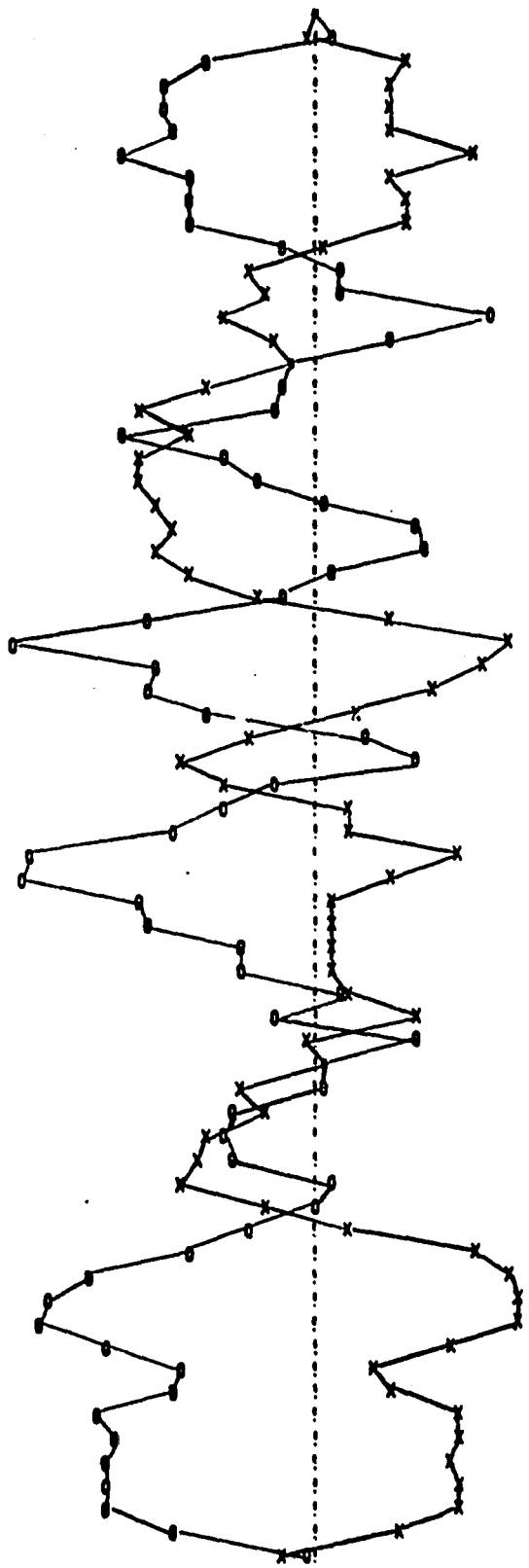
PROTEIN LENGTH = 319

ANALYSIS LENGTH = 5

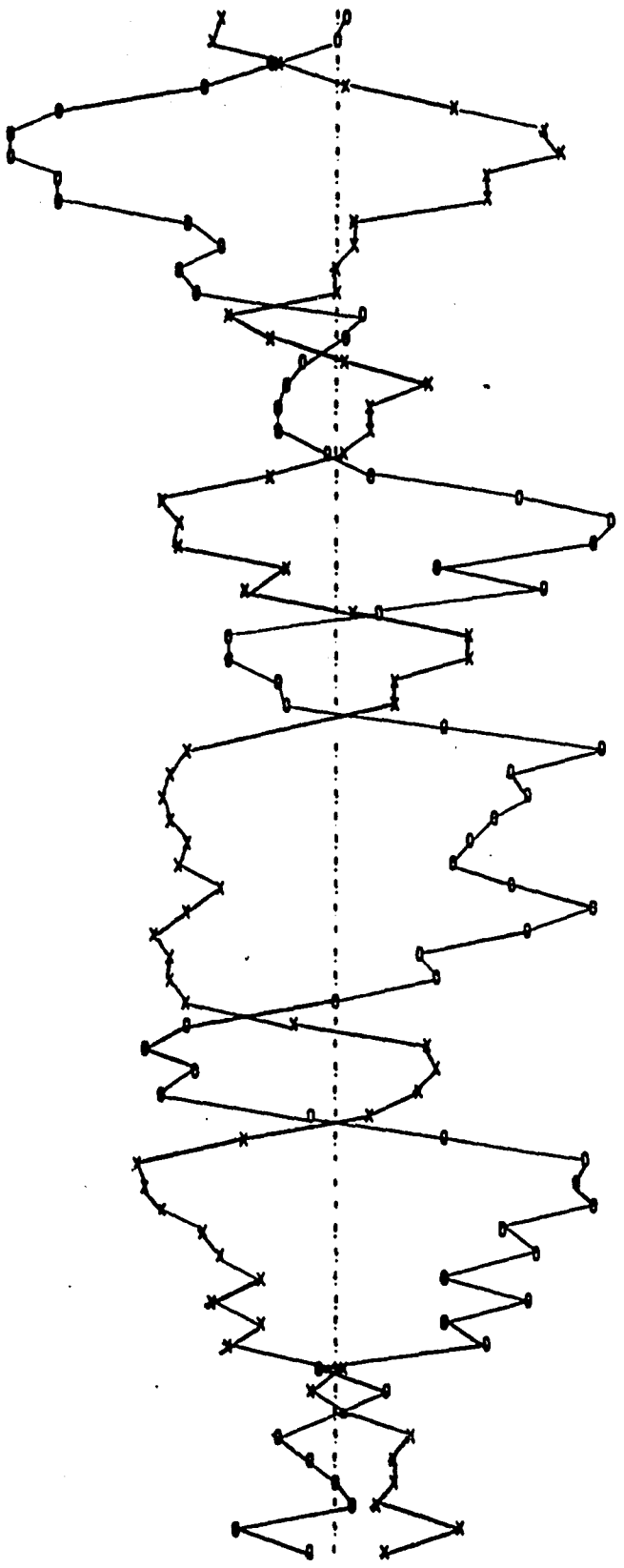


COUNT		AMINO ACID	AVE. HW	AVE. HAD
1	V	V.A.L	0	4.42
2	G	G.L.Y	.2	3.94
3	C	C.Y.S	.8	3.12
4	D	A.S.P	.92	2.48
5	N	N.I.S	.92	2.28
6	A	A.L.A	1.62	2.02
7	R	A.R.G	1.36	2.56
8	T	T.H.R	.56	3.96
9	R	A.R.G	.56	3.96
10	R	A.R.G	- .12	4.72
11	I	I.L.E	-1.82	4.44
12	C	C.Y.S	- .86	4.86
13	T	T.H.R	-1.16	4.1
14	T	T.H.R	- .48	5.54
15	V	V.A.L	- .34	5.5
16	C	C.Y.S	- .54	5.2
17	F	P.H.E	- .28	4.52
18	D	A.S.P	- .14	4.72
19	S	S.E.R	- .48	5.24
20	F	P.H.E	- .74	5.1
21	S	S.E.R	- .44	5.06
22	L	L.E.U	- .58	5.1
23	S	S.E.R	- .16	4.18
24	P	P.R.O	- .72	4.98
25	C	C.Y.S	- .12	4.52
26	T	T.H.R	0.98	3.88
27	S	S.E.R	.88	3.94
28	F	P.H.E	.88	3.94
29	D	A.S.P	.58	3.88
30	T	T.H.R	.84	3.6
31	G	G.L.Y	- .24	4.64
32	S	S.E.R	- .74	5.26
33	P	P.R.O	- .76	4.74
34	S	S.E.R	- .72	4.34
35	I	I.L.E	-1.28	5.08
36	F	P.H.E	-1.28	4.92
37	G	G.L.N	-1.14	5.12
38	G	G.L.N	-1.48	6.36
39	F	P.H.E	-2.88	7.8
40	L	L.E.U	-1.94	8
41	L	L.E.U	-1.58	6.94
42	F	P.H.E	-1.58	7.1
43	L	L.E.U	-1.18	6.92
44	L	L.E.U	- .9	6.84
45	P	P.R.O	.86	4.52
46	I	I.L.E	- .84	5.2
47	A	A.L.A	.32	3.98
48	T	T.H.R	.16	4
49	K	L.Y.S	.3	3.96
50	A	A.L.A	- .44	5.64

51	P	P.R.D	.04	4.5
52	H	H.E.T	- .04	4.68
53	S	S.E.R	.02	3.4
54	I	I.L.E	.46	2.94
55	K	L.Y.S	.46	2.94
56	T	T.H.R	.46	3.02
57	R	A.R.G	1.34	2.46
58	N	N.I.S	.46	3.22
59	I	I.L.E	.8	3.16
60	E	G.L.U	.8	3.16
61	D	A.S.P	.1	4.22
62	T	T.H.R	- .5	4.04
63	N	A.S.N	- .36	4.0
64	I	I.L.E	- .76	4.4
65	A	A.L.A	- .34	5.32
66	G	G.L.Y	- .2	4.26
67	S	S.E.R	- .08	4.16
68	I	I.L.E	-1.4	4.08
69	S	S.E.R	-1	2.48
70	N	A.S.N	-1.42	3.56
71	W	T.R.P	-1.46	3.94
72	Y	T.Y.R	-1.28	4.66
73	N	A.S.N	-1.18	5.66
74	I	I.L.E	-1.32	5.72
75	P	P.R.D	-1.04	4.68
76	F	P.H.E	- .44	4.22
77	L	L.E.U	.66	2.78
78	H	H.I.S	1.62	1.34
79	T	T.H.R	1.42	2.82
80	K	L.Y.S	1.04	2.7
81	R	A.R.G	.36	3.34
82	D	A.S.P	- .54	5.08
83	V	V.A.L	-1.08	5.6
84	Y	T.Y.R	- .74	4.86
85	T	T.H.R	.32	3.54
86	V	V.A.L	.3	3.84
87	S	S.E.R	1.2	1.5
88	Q	G.L.N	.68	1.42
89	K	L.Y.S	.14	2.64
90	H	H.I.S	.14	2.74
91	E	G.L.U	.14	3.74
92	Y	T.Y.R	.14	3.74
93	F	P.H.E	.3	4.04
94	D	A.S.P	.86	4.12
95	A	A.L.A	- .84	5.66
96	E	G.L.U	.1	4.6
97	V	V.A.L	- .6	4.66
98	S	S.E.R	- .38	3.68
99	V	V.A.L	- .9	3.6
100	Q	G.L.N	- .96	3.66
101	N	N.I.S	-1.1	4.72
102	T	T.H.R	- .4	4.58
103	Y	T.Y.R	.28	3.82
104	I	I.L.E	1.34	3.18
105	A	A.L.A	1.42	2.14
106	K	L.Y.S	1.72	1.7
107	R	A.R.G	1.72	1.58
108	R	A.R.G	1.18	2.3
109	T	T.H.R	.5	3.86
110	G	G.L.Y	.64	3.02
111	R	A.R.G	1.24	2.2
112	S	S.E.R	1.24	2.4
113	T	T.H.R	1.18	2.26
114	S	S.E.R	1.26	2.32
115	R	A.R.G	1.26	2.32
116	E	G.L.U	.72	3.84
117	P	P.R.D	- .24	4.48



118	G	G.L.Y	- .92	4.42
119	S	S.E.R	-1	4.56
120	S	S.E.R	- .44	3.84
121	L	L.E.U	.87	3.12
122	W	T.R.P	1.04	1.48
123	T	T.N.R	1.76	.96
124	R	A.R.G	1.9	.92
125	R	A.R.G	1.3	1.5
126	R	A.R.G	1.3	1.5
127	N	A.S.N	.2	2.94
128	S	S.E.R	.16	3.32
129	P	P.R.O	0	2.84
130	R	A.R.G	.84	3
131	F	P.H.E	- .9	4.8
132	P	P.R.O	- .5	4.42
133	N	H.I.S	.1	4.16
134	S	S.E.R	.8	4.02
135	I	I.L.E	.28	3.94
136	A	A.L.A	.28	3.94
137	K	L.Y.S	.88	4.42
138	K	L.Y.S	- .52	4.88
139	Y	T.Y.R	-1.42	6.5
140	I	I.L.E	-1.32	7.5
141	V	V.A.L	-1.32	7.34
142	P	P.R.O	- .42	5.6
143	V	V.A.L	- .72	6.76
144	L	L.E.U	.18	5.02
145	L	L.E.U	1.14	3.38
146	R	A.R.G	1.14	3.38
147	V	V.A.L	.54	3.96
148	R	A.R.G	.54	3.96
149	R	A.R.G	- .36	5.7
150	L	L.E.U	-1.26	7.44
151	P	P.R.O	-1.36	6.44
152	V	V.A.L	-1.44	6.62
153	V	V.A.L	-1.34	6.28
154	V	V.A.L	-1.24	5.94
155	Y	T.Y.R	-1.3	5.84
156	T	T.N.R	- .94	6.46
157	C	C.Y.S	-1.22	7.34
158	C	C.Y.S	-1.48	6.58
159	L	L.E.U	-1.38	5.44
160	A	A.L.A	-1.38	5.6
161	L	L.E.U	-1.24	4.54
162	Y	T.Y.R	- .28	2.9
163	N	H.I.S	.78	2.46
164	I	I.L.E	.08	3.02
165	Q	G.L.N	.74	2.66
166	R	A.R.G	.34	4.26
167	D	A.S.P	- .76	5.7
168	G	G.L.Y	-1.66	7.24
169	F	P.H.E	-1.6	7.14
170	I	I.L.E	-1.46	7.34
171	F	P.H.E	-1.1	6.36
172	V	V.A.L	- .96	6.72
173	S	S.E.R	- .6	5.7
174	L	L.E.U	-1.02	6.62
175	G	G.L.Y	- .6	5.7
176	I	I.L.E	- .86	6.16
177	S	S.E.R	.1	4.36
178	L	L.E.U	- .16	5.04
179	S	S.E.R	.1	4.66
180	M	M.E.T	.64	3.94
181	R	A.R.G	.54	4.3
182	C	C.Y.S	.54	4.5
183	A	A.L.A	.28	4.74
184	R	A.R.G	1.08	3.48
185	L	L.E.U	.48	4.3



186	E	G.L.N	.76	3.42
187	L	L.E.U	- .32	3.94
188	R	A.R.G	- .44	3.74
189	G	G.L.Y	-1.36	5
190	T	T.N.R	- .76	4.18
191	W	T.R.P	- .76	4.18
192	F	P.N.E	- .44	5.24
193	A	A.L.A	- .16	5.22
194	R	A.R.G	-2.49	4.16
195	T	T.N.R	- .6	4.98
196	I	I.L.E	- .08	6.02
197	C	C.Y.S	.08	4.22
198	N	A.S.N	.08	4.22
199	G	G.L.Y	- .32	5.02
200	I	I.L.E	- .78	5.64
201	R	A.R.G	- .72	5.58
202	C	C.Y.S	- .72	5.58
203	I	I.L.E	- .02	5.92
204	Y	T.Y.R	.14	4.12
205	V	V.A.L	1.2	3.48
206	R	A.R.G	1.42	2.5
207	V	V.A.L	.72	3.76
208	R	A.R.G	.92	3.28
209	R	A.R.G	- .84	4.92
210	T	T.N.R	-1	4.56
211	A	A.L.A	-1.12	7.2
212	A	A.L.A	-1.32	7.48
213	L	L.E.U	-1.38	8.22
214	L	L.E.U	-1.72	8.82
215	C	C.Y.S	-1.36	7.2
216	V	V.A.L	- .56	6
217	I	I.L.E	- .34	5.82
218	F	P.N.E	- .18	4.62
219	G	G.L.Y	- .18	4.62
220	D	A.S.P	- .38	5.2
221	T	T.N.R	-1.28	6.74
222	C	C.Y.S	-1.7	7.42
223	F	P.N.E	-1.5	6.84
224	C	C.Y.S	-1.36	7.2
225	V	V.A.L	-1.46	7.54
226	F	P.N.E	-1.52	7.44
227	G	G.L.Y	-1.12	6.26
228	I	I.L.E	-1.06	6.16
229	V	V.A.L	- .8	5.42
230	L	L.E.U	- .86	5.52
231	N	N.I.S	-1	5.32
232	S	S.E.R	-1.2	6.8
233	A	A.L.A	- .66	6.28
234	L	L.E.U	- .82	6.3
235	F	P.N.E	- .66	6.06
236	V	V.A.L	- .12	4.82
237	E	G.L.U	.78	3.88
238	N	N.E.T	.78	3.88
239	C	C.Y.S	.58	2.44
240	G	G.L.N	.42	2.68
241	R	A.R.G	.98	2.48
242	E	G.L.U	- .07	3.12
243	Y	T.Y.R	- .68	3.74
244	L	L.E.U	.38	3.1
245	R	A.R.G	.64	2.72
246	Y	T.Y.R	- .46	4.16
247	G	G.L.Y	- .5	4.96
248	R	A.R.G	-1.18	4.66
249	A	A.L.A	-1.78	5.44
250	F	P.N.E	-1.62	4.9
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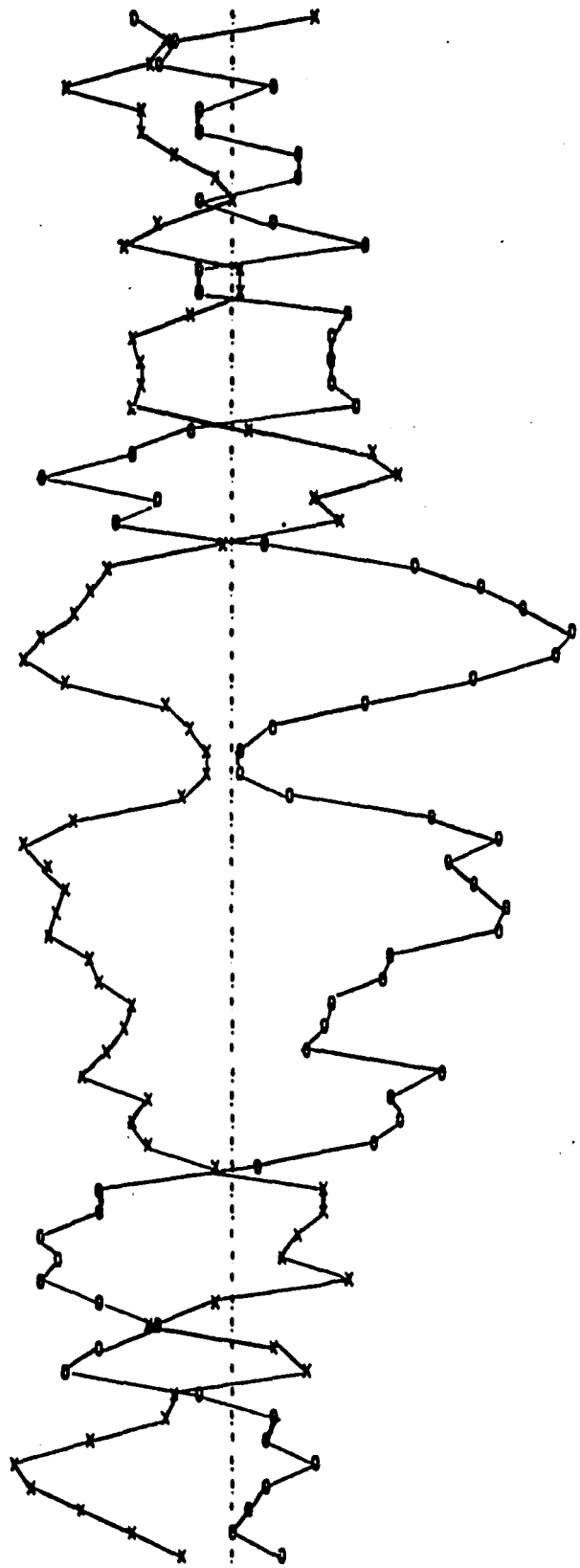
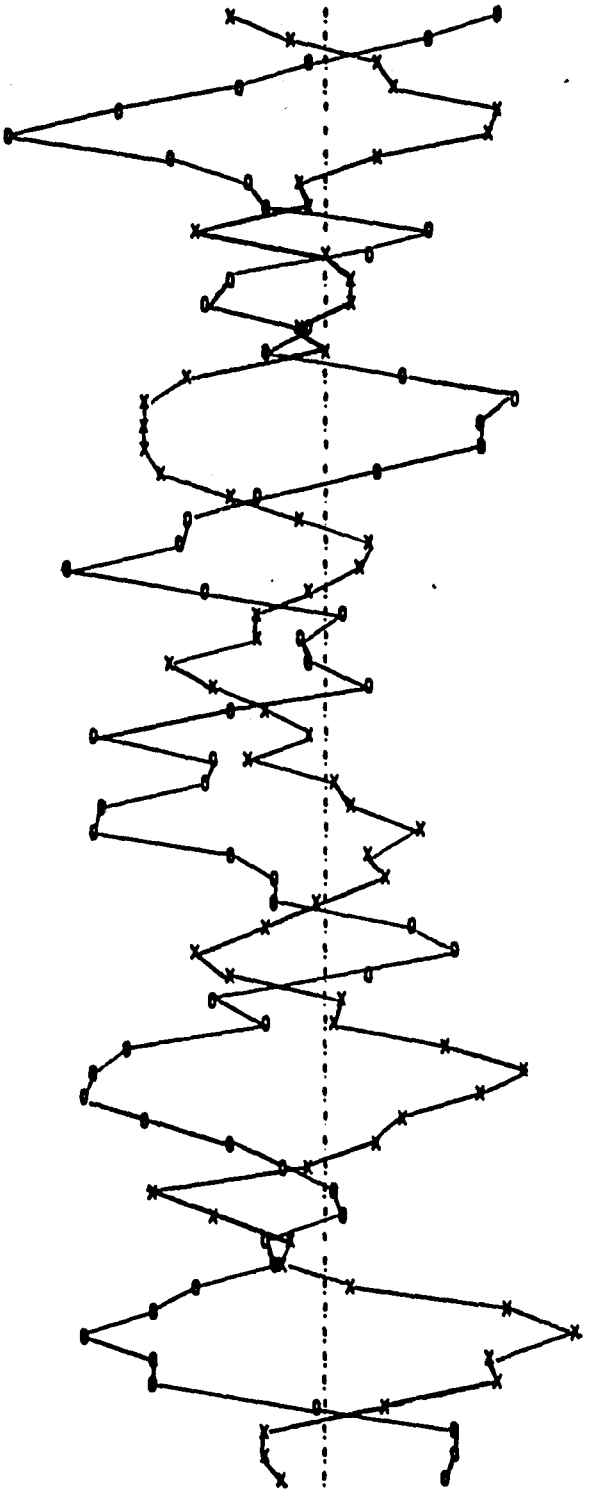
252	W	T.R.P	- .02	4.54
253	P	P.R.O	- .4	5.1



254	S	S.E.R	- .76	6.22
255	A	A.L.A	- .22	5.6
256	A	A.L.A	.98	4.34
257	M	M.E.T	.58	3.44
258	I	I.L.E	1.44	2.38
259	R	A.R.G	1.34	1.22
260	R	A.R.G	.44	2.96
261	P	P.R.D	- .16	3.78
262	R	A.R.G	- .1	3.92
263	Y	T.Y.R	-1	5.44
264	V	V.A.L	.86	5.02
265	G	G.L.Y	.26	3.54
266	S	S.E.R	.26	3.3
267	V	V.A.L	- .16	4.38
268	R	A.R.G	.84	3.9
269	H	H.I.S	-1.86	5.34
270	P	P.R.D	-1.46	6.32
271	I	I.L.E	-1.42	6.14
272	A	A.L.A	-1.42	6.14
273	F	P.H.E	-1.28	5.68
274	F	P.H.E	- .74	3.84
275	Q	G.L.N	- .18	3.12
276	I	I.L.E	.38	3.84
277	Q	G.L.N	.28	1.98
278	Q	G.L.N	- .12	3.32
279	S	S.E.R	- .52	4.76
280	K	L.Y.S	- .54	4.24
281	Y	T.Y.R	-1.24	4.38
282	L	L.E.U	- .08	5
283	L	L.E.U	- .48	3.56
284	Q	G.L.N	- .08	2.12
285	H	H.I.S	- .62	3.36
286	A	A.L.A	.08	3.3
287	Q	G.L.N	.22	2.24
288	Q	G.L.N	.78	2.16
289	F	P.H.E	.38	3.6
290	E	G.L.U	.52	3.96
291	Q	G.L.N	- .03	3.96
292	K	L.Y.S	- .44	5.4
293	L	L.E.U	-1.04	5.06
294	I	I.L.E	- .76	4.98
295	Q	G.L.N	.2	3.38
296	L	L.E.U	.08	3.94
297	P	P.R.D	1.04	2.5
298	T	T.H.R	1.44	2.12
299	D	A.S.P	1.26	2
300	T	T.H.R	.46	2.62
301	D	A.S.P	.44	3.6
302	E	G.L.U	- .1	4.12
303	Y	T.Y.R	-1.38	4.64
304	G	G.L.Y	- .86	4.72
305	V	V.A.L	- .26	3.9
306	S	S.E.R	- .32	3.96
307	U	T.R.P	.22	3.24
308	S	S.E.R	1.5	2.72
309	R	A.R.G	2.04	2
310	I	I.L.E	1.36	2.76
311	R	A.R.G	1.42	2.7
312	E	G.L.U	.52	4.44
313	R	A.R.G	- .44	5.08
314	T	T.H.R	- .44	5.08
315	V	V.A.L	- .3	5.04
316	V	V.A.L		
317	L	L.E.U		
318	R	A.R.G		
319	S	S.E.R		



Woods (38) with a window of 5 amino acids. The product of expression of the 1.2 kb fragment DNA appeared to contain several hydrophobic stretches. However, it was difficult to infer whether the enzyme was membrane-associated or not since it did not have stereotypic hydrophobic regions like those found in other membrane-associated proteins.

DISCUSSION

A gene presumably coding for a β-glucosidase was isolated from a Bacillus cereus/Cellulomonas sp. hybrid. The hybrid bacterium was obtained through protoplast fusion of a Bacillus cereus tetracycline resistant mutant and a Cellulomonas sp. expressing cellulolytic activity (29).

Early attempts to clone the gene encoding a protein which is responsible for β-glucosidase activity into E. coli plasmid pBR322 by using BamHI endonuclease were unsuccessful. However, four clones with β-glucosidase activity were found in a gene library constructed with pBR322 and a HindIII digestion of the DNA from the hybrid organism. One clone (pGS2) containing a 1.2 kb fragment was characterized further. E. coli JM83 host cells are unable to utilize cellobiose as a carbon source. Hence, β-glucosidase specifying plasmids were selected by growth of the transformed cells on minimal cellobiose medium. The β-glucosidase enzyme activity of pGS2 transformed E. coli was further assayed with the chromogenic substrate P-nitrophenyl β-D-glucopyranoside (PNPG). That the 1.2 kb DNA fragment was indeed responsible for the β-glucosidase activity was shown by Tn5 transposon mutagenesis of the gene fragment. When Tn5 was inserted in the fragment, the gene lost its ability to code for a protein having the enzyme activity, resulting in the inability of JM83 with plasmid to grow on cellobiose.

An attempt to isolate the β-glucosidase gene from Cellulomonas sp. was carried out. Genomic DNA was digested with endonuclease BamHI and cloned into BamHI digested pBR322. No clones were obtained on minimal cellobiose medium plates after transformation of JM83 with the pBR322

clones. One possible reason for the lack of success is that Cellulomonas sp. genomic DNA is highly methylated (Deobagkar, D.N., personal communication). This might have caused the DNA to be poorly digested with the BamHI restriction enzyme (manufacturer, B.R.L., suggests that methylated DNA is not cleaved by BamHI). Further studies will be required to overcome the difficulties involved in direct cloning of the β-glucosidase gene from Cellulomonas sp.

Southern transfer and dot blot hybridization of pGS2 showed homology with Bacillus cereus/Cellulomonas sp. hybrid but not with the Cellulomonas sp. Since Cellulomonas sp. is one of the parent organisms of the hybrid and has cellulolytic activity, it was expected to possess DNA sequence homology. However, the failure of the hybridization reaction with Cellulomonas DNA is not understood at the moment. Hybridization of pGS2 with B. cereus genomic DNA was not tested on the presumption that B. cereus did not grow on cellobiose. However, the lack of growth of B. cereus on cellobiose may be due to inability of transport of cellobiose into organism despite the presence of gene for β-glucosidase. Hence, the origin of this particular 1.2 kb fragment in the hybrid can be determined only by further experiments.

The 'aryletherase' from Erwinia sp. isolated by Y. Chon (15) could release p-nitrophenol from PNPG but it lacked the ability to grow on cellobiose. Thus it was thought that the Erwinia sp. possesses an enzyme which recognizes molecules containing aromatic nuclei. The gene coding for the 'aryletherase' was cloned into pBR322 (designated as pNCI) by K.E. Narva (68). E. coli CS412 transformants carrying pNCI

expressed 'aryletherase' activity. Thus although this 'aryletherase' also breaks down PNPG, the mode of enzyme reaction is probably different from the presumptive β-glucosidase specified by plasmid pGS2. Comparison of restriction sites in the restriction enzyme maps of cloned DNAs (pGS2 and pNCI) did not show any similarity. The hydrophilicity and hydrophobicity of proteins deduced from the nucleotide sequences of both clones were also different. Furthermore, in order to show PNPG activity of 'aryletherase', the E. coli with pNCI did not require a pretreatment with surfactants. Hence, this enzyme is possibly present in periplasmic area. Initial amino acids of 'aryletherase' are hydrophobic, consistent with the possibility that the enzyme is secreted to periplasmic area. In contrast, the E. coli with pGS2 required a pretreatment with cetyl trimethyl ammonium bromide (CTAB) to show PNPG activity. This result suggests a cytoplasmic location of pGS2 specified β-glucosidase. This is also consistent with the hydrophilicity pattern of the presumptive β-glucosidase peptide (i.e., the amino acid domains are not typical for a membrane associated protein).

Properties of the purified enzyme from Candida pelliculosa were studied and the nucleotide sequence of this eukaryotic β-glucosidase gene was also determined by Kohchi and Toh-e (48) . They found that the putative protein encoded by this gene had hydrophobic amino acids at its N-terminal region, resembling a signal sequence, and in addition had 19 potential glycosylation sites. An analysis of hydropathy index of the proposed product of the cloned 1.2 kb fragment (pGS2) indicates no hydrophobic region similar to a signal peptide.

Nucleotide sequence of cloned DNA fragment revealed that this insert is 1188 bp in length. The DNA sequence data indicate that pGS2 contains a presumptive β-glucosidase gene but the 3' end of the gene is truncated (Figure 17). The orientation of the cloned DNA fragment in pBR322 has been determined. In this orientation, the presumptive gene transcribed in the same direction as the *tet* gene. The open reading frame of the proposed peptide terminates in the vector. This would add 8 amino acids to the 319 encoded by the insert, and the carboxyl terminus of the peptide encoded by pBR322 would be the following: Phe-Asn-Ala-Val-Val-Tyr-His-Ser.

Search for possible promoters for the presumptive β-glucosidase gene was carried out with pBR322 promoters. pBR322 contains P1, P3 (for ampicillin resistant gene), P2 (for tetracyline resistant gene) (14), RNAI, primer, and P4 promoters (36, 73). But none of these promoters has the correct orientation for transcription of this presumptive β-glucosidase gene. Hence, this inserted DNA fragment must contain a promoter that initiates transcription of the presumptive β-glucosidase gene. When promoter search was carried out by computer with the program TARGSEARCH written by Mulligan *et al.* (67), the strongest putative promoter was inside the coding region of the proposed β-glucosidase gene (-35 region at 423 and -10 region at 443). However, this presumptive promoter does not correlate with any of the possible large open reading frames. A possible promoter with weaker homology with the *E. coli* concensus sequence was located proximal to the start of the aforementioned open reading fram (see Figure 16). As a promoter, this sequence would be predicted to function only weakly.

Sequence analysis revealed 12 short open reading frames and two large imcomplete reading frames. The larger one of the incomplete reading frames is considered to be the best possible coding region for a β-glucosidase gene. The translational start site of this incomplete reading frame is 227 bases from the 5' HindIII end of the 1.2 kb fragment. The DNA sequence at -18 from the ATG initiation codon is AAAGGAGG representing the Shine-Delgarno region. (It is considerably farther from ATG than normal E. coli ribosome binding site, consequently translation initiation is likely to be inefficient.)

Low β-glucosidase activity found from this clone may be also attributed to the altered sequence at the carboxyl end of the gene product. Perhaps the 3' end of the gene is necessary for proper expression of the β-glucosidase gene.

The codon usage in the presumptive β-glucosidase gene was examined (Table 7). No bias of codon usage in the β-glucosidase is observed, since 60 out of 61 potential amino acid codons are used. Preference of A and T to G and C or G and C to A and T at the third letter of the codons in the β-glucosidase gene was not shown (A and T:177, G and C: 142). Since Bacillus cereus was one of the parental organisms of the fusion hybrid and the fusion hybrid appeared to be similar to Bacillus sp., codon usage in this β-glucosidase gene was compared with the 5' terminus of the Bacillus licheniformis α-amylase gene (94). CUC codon for leucine which was not used in β-glucosidase gene was also rare (used only once) in this α-amylase gene of Bacillus licheniformis. ACG, AAU, and GCA codons were the most frequently used codons for threonine, asparagine, and alanine, repectively, in both organisms and also CAU

Table 7. Codon usage in the presumptive β-glucosidase gene.

Phe	UUU:10	Thr	ACU:5	Cys	UGU:6
	UUC:11		ACC:3		UGC:7
Leu	UUA:1		ACA:6	Trp	UGG:5
	UUG:9		ACG:8		
Leu	CUU:10	Ala	GCU:5	Arg	CGU:6
	CUC:-		GCC:3		CGC:1
	CUA:2		GCA:6		CGA:7
	CUG:4		GCG:4		CGG:7
Ile	AUU:4	Tyr	UAU:6	Ser	AGU:4
	AUC:8		UAC:7		AGC:5
	AUA:12			Arg	AGA:10
Met	AUG:4				AGG:5
Val	GUU:5	His	CAU:6	Gly	GGU:4
	GUC:8		CAC:4		GGC:2
	GUA:8	Glu	CAA:7		GGA:7
	GUG:3		CAG:7		GGG:1
Ser	UCU:9	Asn	AAU:4		
	UCC:2		AAC:1		
	UCA:1	Lys	AAA:6		
	UCG:6		AAG:3		
Pro	CCU:3	Asp	GAU:7		
	CCC:1		GAC:3		
	CCA:4	Glu	GAA:6		
	CCG:6		GAG:4		

codon for histidine was more dominant than CAC codon in both genes.

The mechanism of hydrolysis of β-glucosidase is not clearly understood. β-glucosidase from Escherichia adecarboxylata was found to be a membrane-associated enzyme (7) and it required phosphate and magnesium ions to express its activity. It also requires heat stable and non-dialyzable factors from the bacterial cytoplasm. The β-glucosidase from the 1.2 kb fragment appears to be hydrophobic but it is difficult to infer whether the enzyme is membrane-associated as it does not have stereotypic hydrophobic regions like those found in other membrane proteins (Figure 19).

At the present time, it is not possible to produce β-glucosidase in large quantities from the cloned organisms. If this β-glucosidase gene is inserted into expression vectors with more efficient promoters, it may be possible to increase the quantity of the enzyme produced. Recombinant DNA technology may also yield insights into the active site of the enzyme. In addition, in vitro mutagenesis of the cloned gene may make it possible to mutate the gene such that the enzyme overcomes the glucose inhibition. The availability of separate clones for each enzyme of the cellulase complex will provide flexibility for the cellulose degradation processes and will yield more efficient systems for the conversion of cellulose to glucose. These studies on the molecular cloning of a gene for β-glucosidase from a cellulolytic hybrid of B. cereus and Cellulomonas sp. are an initial attempt toward developing an efficient cellulolytic bioprocess.

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VITAE

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DOCTORAL EXAMINATION AND DISSERTATION REPORT

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Major Field: Microbiology

Title of Dissertation: Studies on the Molecular Cloning of a Gene for β-glucosidase from a Bacillus cereus/Cellulomonas sp. Hybrid Bacterium Derived through Protoplast Fusion.

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